The Effects of Weaning Stress on the Serum Protein Profile of Calves: A Proteomic Analysis

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ABSTRACT

Studies in animals and humans link both physical and psychological stress with an increased rate and severity of infections and onset of diseases. Stress is a very broad and complex topic. It can be defined as a condition occurring in response to adverse external influences capable of affecting physical health which leads to activation of a stress response in the body. There are two prominent stress responsive systems: the hypothalamic-pituitary-adrenal axis and the sympathetic adrenomedullary axis. These systems are responsible for the majority of the changes in the body, which occur in response to stress. Stress has been linked to many detrimental effects in cattle including immune suppression, increased disease susceptibility and decreased reproduction. These cause huge economic losses to the cattle industry every year. Weaning has been identified as one of the main stressors implicated in these negative effects. For this reason it is important to be able to identify animals stressed by weaning and do so using samples which are easily obtainable and useful for future diagnostic purposes. We hypothesize that weaning will cause sufficient stress in cattle to alter protein profiles in serum, which can be used to identify this type of stress. To do this we employed proteomic methodologies including two-dimensional gel electrophoresis and mass spectrometry to compare an abrupt weaned group of calves to a never weaned group and a previously weaned group (preconditioned). We have included a preconditioned group to examine the differences between this group and animals which have never been weaned. Preconditioned animals are typically used as a control group in weaning studies. A total of 83 distinct protein bands were identified after image analysis. Out of 83 protein bands, we found 9 spots which were significantly different in abundance

among the treatment groups. Two out of 9 spots were significantly different between the abrupt weaned and the never weaned groups. Five protein bands were also found to be significantly different between the abrupt weaned group and the preconditioned group. Five protein bands were found to be significantly different between the never weaned group and the preconditioned group. Identification of these proteins, however, had limited success since the bovine protein database is not as extensive as that for humans or mice. Among the proteins identified were alpha-1-acid glycoprotein and collagen precursor. The differences in intensities found between the abrupt weaned group and the never weaned group may be useful as markers of calves going through weaning stress. We have also seen that animals who have undergone weaning and through the stress associated with that event are not exactly the same as animals which have never been weaned. This has implications to research where a preconditioned group is used as a control rather than a never weaned group. Despite the limitations of the methodology used for the current system, the overall results revealed specific changes in serum proteins which were associated with abrupt weaned animals. Future studies can be planned to determine the specificity of these protein changes and possibly identify the molecular basis of stress dependent disease susceptibility.

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2D-GE	two-dimensional gel electrophoresis
ACTH	adrenocorticotropin hormone
AGP	alpha-1-acid glycoprotein
ANOVA	analysis of variance
AW	abrupt weaned group
BDNF	brain-derived neurotrophic factor
BHV-1	bovine herpesvirus-1
BRD	bovine respiratory disease
BSA	bovine serum albumin
CAs	catecholamines
CD40L	CD40 ligand
CHCA	α-cyano-4-hydoxycinnamic acid
CHD	coronary heart disease
CNS	central nervous system
CRF_1	CRH receptor
CRH	corticotropin releasing hormone
D	drinking
DEX	dexamethasone
DC	dendritic cell
DFD	dark, firm and dry meat
E	epinephrine
Eat	eating

GR	glucocorticoid receptor
HPA	hypothalamic-pituitary-adrenal axis
IEF	isoelectric focusing
IL	interleukin
IFN- γ	interferon-γ
IR	insulin resistance
IRS-1	insulin receptor substrate-1
IRS-2	insulin receptor substrate-2
JIA	juvenile idiopathic arthritis
L	lying
LC/NE	locus coeruleus/norepinephrine system
LR	lying while ruminating
MALDI-TOF	matrix assisted laser desorption/ionization-time of flight
MR	mineralocorticoid receptor
mRNA	messenger RNA
MS	mass spectrometry
N	nursing
NE	norepinephrine
NGF	nerve growth factor
NK cells	natural killer cells
NT-3	neurotrophin-3
N:L	neutrophil: lymphocyte

NW	never weaned group
PBLs	peripheral blood leukocytes
PBMCs	peripheral blood mononuclear cells
PC	preconditioned group
PLAs	platelet-leukocyte aggregates
PSGL-1	P-selectin glycoprotein ligand-1
PVN	paraventricular nucleus
RA	rheumatoid arthritis
S	standing
SAM	sympathetic adrenomedullary system
SELDI	surface enhanced laser desorption/ionization
SH-2	Src homology-2 domain
SST	serum-separator tubes
SNS	sympathetic nervous system
SR	standing while ruminating
T2D	diabetes mellitus type 2
Th	t-helper cells
THI	temperature-humidity index
TNF-α	tumor necrosis factor alpha
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone
VOX	vocalizations
W	walking

1.0 INTRODUCTION

Stress is a very broad concept and difficult to define in a concise way which captures all of its connotations. Griffin (1989) reports that in the early 20th century Hans Seyle proposed the general adaptation syndrome which provided the first comprehensive biological theory of stress. In a veterinary context this was identified as an abnormal or extreme adjustment in the physiology of the animal to cope with adverse effects of its environment and management (Fraser *et al.* 1975). It is clear that stress can be defined in many different ways depending upon the objectives or perspective of the researcher. For our purposes, stress is defined as a condition occurring in response to adverse external influences capable of altering physiological responses. Many types of stressors can contribute to this condition including psychological stressors and physical stressors. In situations where an individual is crowded, isolated, transported or introduced to a new environment, it can experience psychological stress. In circumstances where an individual is exposed to extreme temperatures or forced exercise, physical stress can be induced. In many cases, a stressor can be composed of both a psychological component as well as a physical component. Such is the case when exposed to painful situations, restraint or during weaning.

There are two main systems that respond to stress in the body: the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic adrenomedullary

(SAM) system (Van de Kar *et al.* 1999; Elenkov *et al.* 2000). Both act to prepare the body for what is commonly known as the fight or flight response. Since the body perceives a stressor as something which decreases the probability of survival, the fight or flight response is necessary to combat it. These stress responsive systems act through a cascade of stress hormones which activate or inhibit the appropriate responses (Elenkov *et al.* 2000). Such responses include increased cardiovascular output, decreased appetite, increased energy to the brain and muscles as well as changes in immune function (Stratakis *et al.* 1995; Habib *et al.* 2001). These responses are important when survival is at risk but, unfortunately, their effects may not always benefit the body.

Many harmful effects of stress have been reported in the literature for both humans and other animals. For humans, stress contributes to cardiovascular disease, learning impairment, and insulin resistance among other things (Straub *et al.* 2005; Black 2006; Brydon *et al.* 2006). For animals such as cattle, reproduction and fertility are negatively affected by heat stress (Wolfenson *et al.* 2000). Stress has also been implicated in decreased weight gain and quality of meat (Trunkfield *et al.* 1990; Shaefer *et al.* 1997). The aforementioned effects have an impact on the health of the individual as well as industries pertaining to the production of animals; however, the effects of stress on the immune system have by far the greatest impact of all (Yang *et al.* 2000; Padgett *et al.* 2003). Stress has been shown to alter immune function in ways which can lead to increased susceptibility to infection as well as severity of disease (Yang *et al.* 2000). This has been demonstrated in many species including humans, cattle and mice (Glaser *et al.* 1992; Galyean *et al.* 1999; Mineur *et al.* 2006). It is important to study

stress and how it causes its many effects in order to gain a complete understanding of the mechanisms and to help prevent or combat these negative repercussions.

In order to study stress, there must be a clear and simple way of identifying it. The term biomarker denotes a substance used as an indicator of a biologic state. This can be very useful when identifying and defining a condition such as stress. It has been observed that cortisol concentrations, both serum cortisol as well as salivary, change in response to stressful conditions (Beerda *et al.* 1996). This would suggest that it could be used as a biomarker for stress. Many studies have used cortisol as a biomarker of stress. Cortisol levels increased above normal concentrations are a good indicator that the animal is considered to be stressed (Grandin 1997). However, when studying certain types of stressors, such as weaning in cattle, cortisol is unreliable when samples are collected once a day (Hickey *et al.* 2003). To detect significant changes in concentration, cortisol levels must be monitored more frequently (Queyras *et al.* 2004). For example, sampling on an hourly basis may be more revealing but hourly sampling is impractical when dealing with animals such as calves. The extra handling for sample collection can add to the stress of the calf as the restraint and human contact are powerful psychological stressors; therefore, there is a great need for a more practical, reliable and consistent indicator of stress.

Weaning has long been identified as a stressor. This has many implications especially to the cattle industry because it has been linked to stress-related immune suppression (Hickey *et al.* 2003). The alteration in immune function caused by stressors has been shown to lead to increased disease susceptibility (Yang *et al.* 2000). Bovine respiratory disease (BRD) has a huge economic impact due to animal loss, decreased

weight of calves and the cost associated with treating this illness (Fike *et al.* 2006). Weaning is recognized as a major contributing factor to BRD (Galyean *et al.* 1999). It is important to study weaning as a stressor in order to determine its effects and the mechanisms through which it exerts these effects. This knowledge may identify effective strategies to prevent or treat related illnesses. The first step is to discover a novel way of identifying weaning stressed animals perhaps by identifying a group of candidate biomarkers and characterizing their pattern of change. These candidate biomarkers or their pattern could be validated clinically to establish them as important biomarkers and could then be used in future studies as well as to eventually develop a diagnostic tool.

With these goals in mind, the effects of weaning stress were studied in serum from young calves to identify potential candidate biomarkers.

2.0 BACKGROUND

2.1 Types of Stressors

Stressors can be separated into three main types: psychological stressors, physical stressors and a combination of both.

2.1.1 Psychological Stressors

A psychological stressor is something which does not physically affect the body directly but is perceived as stressful or dangerous. The individual perceives it as dangerous or bothersome and in so doing creates a stress response. This type of stressor induces various stress responses in the body and somehow through this exerts its physiological effects. There are numerous examples of this type of stressor identified in the literature. For humans, psychological stress can be caused by work, exams, social situations or any life event that is perceived as a significant change . For animals, psychological stress can be caused by transportation (Arthington *et al.* 2003), handling (Grandin 1997), mixing or isolation (Kelley 1980), threat from a predator and many other situations. Psychological stress has been studied in regards to its physiological effects in cattle (Veissier *et al.* 2001).

2.1.2 Physical Stressors

A physical stressor is one which affects the body directly. This type of stressor also induces stress responses in the body. Included in this category are stressors such as heat and cold stress, limit-feeding and exercise (Kelley 1980). This group of stressors affects humans and animals alike.

2.1.3 Combination Stressors

Some stressors have both a psychological component as well as a physical component. This is the case for stressors such as noise, pain, restraint and weaning (Kelley 1980). Weaning stress, for example, is composed of severing the maternal bond which is a psychological stress and a nutritional change which is a physical stress.

2.2 Stress responsive systems

When an individual experiences a stressful stimulus, acquired sensory information is routed through the thalamus or reticular activating system to the amygdala and sensory cortex (Van de Kar *et al.* 1999; Carrasco *et al.* 2003). The sensory cortex relays this information to the lateral amygdala via the perirhinal cortex directly or the hippocampus then the perirhinal cortex. The amygdala is implicated in endocrine, autonomic and behavioral responses. It innervates both the catecholaminergic nuclei and the raphe nucleus which in turn innervate corticotrophin-releasing hormone neurons located in the hypothalamic paraventricular nucleus.

2.2.1 Hypothalamic-pituitary-adrenal axis

The hypothalamus is the starting point of the hypothalamic-pituitary-adrenal axis. This axis is one of two stress responsive systems responsible for the changes that take place in the body after experiencing stressors. Corticotropin-releasing hormone (CRH) is synthesized by the parvicellular neurons in the paraventricular nucleus of the hypothalamus (Van de Kar *et al.* 1999; Carrasco *et al.* 2003). Vasopressin and oxytocin are synthesized in the magnocellular subdivision of the paraventricular nucleus. CRH and vasopressin are released together into the portal circulation where they stimulate adrenocorticotropin hormone (ACTH) release from the anterior lobe of the pituitary gland. Release of ACTH occurs only 10 seconds after stimulus (Sapolsky 2000; Carrasco *et al.* 2003) and serves to regulate the secretion of glucocorticoids from the adrenal glands. Glucocorticoids play a role in homeostasis, energy metabolism, growth processes as well as immune and brain function (Stratakis *et al.* 1995; Habib *et al.* 2001). They do so through interaction with two glucocorticoid receptor subtypes: glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). MRs are present in limbic neurons and GRs are widespread throughout the brain including negative feedback sites in the hypothalamus and pituitary gland (de Kloet 1995). Both subtypes function as transcription factors which are ligand-dependent (Carrasco *et al.* 2003). An example of a glucocorticoid is cortisol. Stimulation of the adrenal cortex to release cortisol is the end-point of the hypothalamic-pituitary-adrenal axis (HPA). Cortisol goes on to mediate many functions in the body and completes a negative feedback loop at the hypothalamus and pituitary gland.

It has been observed that certain cytokines from an immune response can activate the HPA (Besedovsky *et al.* 1986; Chrousos 1995; Elenkov *et al.* 2000). These cytokines include tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6). How they stimulate the release of CRH and ACTH is unclear but these hormones are present in higher quantities in response to circulating cytokines. Since the hypothalamus and pituitary are protected by the blood-brain barrier, it is thought that their activation is achieved through activation of the peripheral and central nervous systems (Chrousos 1995).

2.2.2 Sympathetic adrenomedullary system

The autonomic nervous system is also activated in response to stressful stimuli. The sympathetic subdivision is responsible for many changes in the body associated with the fight or flight response. This begins in a group of sites in the medulla and pons of the brainstem which are collectively known as the locus coeruleus/norepinephrine system (LC/NE) (Elenkov *et al.* 2000). These sites include noradrenergic cell groups as well as the locus coeruleus of the pons (Fisher 1989). Neurons containing CRH are present in many sites of the brain outside the hypothalamus including the LC/NE system (Fisher 1989; Tsigos *et al.* 2002). Stress stimulates the release of CRH from these neurons although many of the mechanisms are unclear (Tsigos *et al.* 2002). CRH functions as a hormone in the HPA but it has been observed that it can also play the role of neurotransmitter in other locations (Fisher 1989; Fisher *et al.* 1991; Minton 1994). One such location is the LC/NE system. Here CRH serves to activate noradrenergic neurons (Arlt *et al.* 2003), which stimulate the release of norepinephrine (NE) in several

parts of the brain (Elenkov *et al.* 2000). NE is the major neurotransmitter of the sympathetic nervous system (SNS). Elenkov *et al* (2000) reviewed the physiology of the SNS quite simply and concisely. They stated that the SNS begins in the central nervous system (CNS), specifically with cell bodies in the brainstem and spinal cord. From there, preganglionic efferent nerve fibres extend down the spinal cord and exit through thoracic and lumbar spinal nerves, which are known as the thoracolumbar system. These preganglionic fibres end at ganglia located in the paravertebral chains on either side of the spinal column as well as prevertebral ganglia in front of the vertebrae. Postganglionic fibres run from the ganglia and innervate tissue throughout the body. Most of these postganglionic fibres act by releasing NE. Some sympathetic preganglionic fibres do not end in ganglia and extend to glands such as the adrenal medulla. Chromaffin cells in the medulla are innervated by preganglionic nerve terminals, which use acetylcholine as their neurotransmitter. When the SNS is activated, the adrenal medulla is stimulated to release epinephrine (E) and NE although E is released in much greater quantities.

Catecholamines (CAs) are involved in many aspects of the fight or flight response including the release of energy or fuel metabolism, cardiovascular effects such as increased heart rate and blood vessel tone, thermogenesis and immunomodulation (Elenkov *et al.* 2000; Kohm *et al.* 2001; Lundberg 2005). Sympathetic nerve fibres innervate many organs important to the immune system including; spleen, thymus, bone marrow, lymph nodes and mucosa-associated lymphoid tissue (Elenkov *et al.* 2000). Within these organs, the nerve fibres end in what are called varicosities. This is an end of the fibre without cell contact or a synapse. They release NE which diffuses to the

immune cells and interacts with them via adrenoreceptors setting off specific signal transduction pathways within the cells. An example of this is the inhibition of dendritic cell migration, decreased antigen presentation and decreased T helper cell type 1 priming in dendritic cells (DCs) (Chrousos 1995; Maestroni 2005). These processes are mediated through the interaction of NE with β-adrenergic receptors on the DC surface.

The sympathetic adrenomedullary system (SAM) and the HPA work together to ensure homeostasis is maintained. It is not surprising then that they also communicate between each other. Neurons containing CRH run from the paraventricular nucleus (PVN) of the hypothalamus to the brainstem and likewise, neurons containing CAs can be traced from the LC/NE to the PVN along the ascending noradrenergic bundle (Chrousos *et al.* 1992; Elenkov *et al.* 2000). This ensures that activation of one system triggers the activation of the other.

As with the HPA, the sympathetic adrenomedullary system can also be activated by circulating cytokines during an immune response (Chrousos 1995; Elenkov *et al.* 2000). They act as a negative feedback loop between the immune system and the brain. In response to these cytokines, both stress systems are activated and work to inhibit many aspects of the immune response. The presence of this feedback loop may serve to keep immune responses such as inflammation in check and not allow them to get out of control.

Although the physiology of induction of HPA and SAM and the effects of stress are complex subjects and many aspects still need to be explored at length, the aforementioned is a synopsis of the overall knowledge relevant to work in this thesis. Since cattle were used as a model in this work, I would like to introduce the subject of

stress and its effects in terms of cattle followed by a brief overview of the topic in humans.

2.3 Effects on Cattle

The effects of several types of stressors have been studied in relation to cattle. Stress in cattle is very important because it has been linked to decreased reproduction, decreased meat quality, disease susceptibility as in the case of bovine respiratory disease and more (Kelley 1980; Tarrant 1990; Trunkfield *et al.* 1990; Shaefer *et al.* 1997; Fike *et al.* 2006). All of these conditions contribute to huge economic losses in the cattle industry every year (Fike *et al.* 2006). Among the most studied stressors are weaning, heat stress, and transport. Weaning and transportation have been indicated as two of the most important stressors that cattle can experience (Johnson *et al.* 1976; Kelley 1980). There is extensive literature on the effects of transportation on cattle.

2.3.1 Transportation

The effects of all stages of transportation on cattle are well documented in the literature. Transportation stress includes the effects of handling during loading and unloading, removal from familiar environment and placement into a novel environment, mixing with unfamiliar animals, changing temperature, and deprivation of food and water as well as weaning depending on the age of the calves (Trunkfield *et al.* 1990). Stress in general is known to be an immunosuppressant (Kelley 1980). This is also the case for transport stress in cattle. Fike and Spire (2006) reported that cattle which have been transported have a decrease in lymphocytes and an increase in neutrophils in the

blood. They also reported a decrease in antibody responses three days after transport and a decrease in lymphocyte blastogenesis in calves after transport (Fike *et al.* 2006). Perhaps an explanation for the connection between transport and bovine respiratory disease is the effects of this stress on the cells in the lung. Macrophages and lymphocytes in bronchoalveolar fluid from young calves were changed in number and function after short-term transport (Ishizaki *et al.* 2005). Lowered immunity in the lung could contribute to the onset of respiratory diseases.

Transport has been shown to elevate cortisol levels in calves (Johnson *et al.* 1976; Trunkfield *et al.* 1990). Some studies have attempted to break transportation down into its aforementioned components. Loading and unloading have proved to be the most stressful components (Kent *et al.* 1983; Trunkfield *et al.* 1990). This is most likely due to the physical exertion, noise and human contact. Cortisol has been shown to increase during the first two hours of transportation and stabilize over longer trips (Kent *et al.* 1983). This suggests that the most stressful event is loading at the beginning of transport. Even human contact alone raises the heart rate of an animal (Stephens *et al.* 1974). Elevations in glucocorticoids decrease as calves become accustomed to human contact and handling (Crookshank *et al.* 1979). In addition to loading, introduction to a new environment, social mixing, animal density and length of journey have been studied individually and have been associated with the induction of stress and increased cortisol (Trunkfield *et al.* 1990).

It has been reported that transportation has also been shown to reduce live weight, reduce carcass yield and lower meat quality (Shaefer *et al.* 1997). A condition known as dark, firm and dry meat (DFD) occurs with greater frequency after an animal

has been stressed (Trunkfield *et al.* 1990). DFD is caused by muscle glycogen store depletion before death. DFD reduces the quality of meat and therefore, creates economic losses in the beef industry. If there were a method or tool to indicate which animals were stressed before slaughter, then stressed animals could be put aside to regenerate glycogen and reduce the incidence of DFD. Reducing the stress involved in all aspects of transportation would be highly beneficial to the beef industry. It would improve the welfare of the animals as well as reduce economic losses.

2.3.2 Weaning

There has been limited research on the effects of weaning alone on cattle. The majority of the literature focuses on weaning and transportation together since often both of these stressors occur at around the same time in a farm setting. However, abrupt weaning is known to cause stress in calves and is therefore an important area to study further (Kelley 1980). The few studies on weaning alone have identified some interesting effects. It has been observed that weaning alone does not increase cortisol levels in calves (Lefcourt *et al.* 1995; Hickey *et al.* 2003). This would suggest that another method of identifying weaning stress is required since changes in cortisol levels do not appear to apply to weaning stress. Epinephrine and norepinephrine levels were also measured to assess the activation of SAM. Epinephrine levels did not change however, norepinephrine increased compared to controls (Lefcourt *et al.* 1995; Hickey *et al.* 2003). Hickey *et al* (2003) also found that weaning stress altered the neutrophil:lymphocyte (N:L) ratio increasing the proportion of neutrophils. Changes in the N:L ratio is thought to be a potential biological indicator of stress and disease

susceptibility. One detail to note about the study by Hickey *et al* (2003) is that the calves were not only weaned from the dams but were kept in isolation after the abrupt weaning. Since isolation is also a stressor, this may act in part as a confounding variable. Thus, there is a lack of research specifically addressing weaning stress. Further studies are required to augment our knowledge of this stressor and its effects on calves. In addition, since cortisol levels do not appear to change in response to weaning, another marker is required.

2.3.3 Heat Stress in Dairy Cattle

There is a large body of literature on the effects of heat stress on the reproduction of dairy cattle (Wolfenson *et al.* 2000; De Rensis *et al.* 2003; Garcia-Ispierto *et al.* 2006). Heat stress in the summer months lowers the conception rate of lactating dairy cows from 40-60% to 10-20% (Wolfenson *et al.* 2000). This creates huge economic losses to the dairy industry. Heat stress has both direct and indirect effects on cattle reproduction. These direct effects on the reproductive process are considered to be the predominant cause of low conception rates (Wolfenson *et al.* 2000). Indirect effects include redistribution of blood flow among body organs and a reduction in food intake. The direct effects of heat stress include changes in cellular functions of different tissues and parts of the reproductive system (Wolfenson *et al.* 2000). Specifically, it negatively affects the preovulatory follicle, developing embryo in early stages, corpus luteum, uterine endometrium and anterior pituitary. The net result is low fertility and also the loss of fetuses.

In an attempt to measure the amount of heat stress cattle can endure, a calculation called the temperature-humidity index (THI) is used (Garcia-Ispierto *et al.* 2006). This is a measure of heat comfort. Heat stress is defined as a THI over 72 units (Garcia-Ispierto *et al.* 2006). Garcia-Ispierto et al (2006) reported a correlation between a high THI value on days 21-30 of gestation and fetal loss. There are no reports in the literature regarding cortisol measurement during heat stress.

2.3.4 Social Reorganization

Social reorganization of cattle is a psychological stressor (Kelley 1980). As a stressor, it has rarely been studied alone. Only two studies focused on social mixing stress by itself (McVeigh *et al.* 1982; Veissier *et al.* 2001). Vessier *et al* (2001) reported that it is a common farm practice to periodically reorganize cattle into new groups based on weight gain and feed rations. Since this social mixing is a known stressor, this is a questionable practice. It may cause unnecessary negative effects on meat quality and animal welfare. McVeigh *et al* (1982) found that social mixing decreased the muscle glycogen and recovery of glycogen stores was slow. They also observed increases in body temperature and heart rate following social reoganization. After multiple mixes, Veissier *et al* (2001) reported no change in cortisol levels between mixed animals and controls. However, the two treatment groups did differ in their response to dexamethasone injection. Mixed animals showed an increased level of cortisol after dexamethasone injection versus before injection. Behavioral observations were also recorded in this study and the majority of the differences between the mixed animals and controls occurred only after the first mixing. In addition to social mixing in the farm

setting, transportation involves many instances of social reorganization. Therefore, studies on transportation of cattle also include a component of social reorganization.

Important stressors and their impacts on cattle have been reviewed; however, these stressors along with other stressors are also important in humans although the end responses may be different.

2.4 Effects of Stress on Human Health

Stress can also have negative effects on human health. Some very common human disorders may have a causative component of stress. The following are a few examples of how stress can negatively affect the human population.

2.4.1 Cardiovascular Diseases

Stress has been associated with increased risk of cardiovascular diseases such as acute coronary events, atherosclerosis and hypertension. Many studies have made the observation that psychological stress contributes to the development of these diseases; however the mechanisms remain elusive. Atherosclerosis or coronary heart disease is a hardening of the arteries due to plaque formation. The result is a narrowing of the arteries which leads to insufficient blood supply to the organ. In the case of the heart, insufficient blood supply causes myocardial infarction or a heart attack. High fat diets, inactivity and high blood pressure are well known risk factors for coronary heart disease (CHD) but lately connections have been made between psychological stress and CHD (Manuck *et al.* 1988; Allen *et al.* 1995; Rozanski *et al.* 1999; Girod *et al.* 2004; Brydon *et al.* 2006).

One mechanism by which stress is thought to contribute to CHD is hemoconcentration (Allen *et al.* 1995). This process can contribute to many cardiovascular system problems including stroke, myocardial infarction, cardiac ischemia and atherosclerosis. Hemoconcentration is caused by a decrease in blood plasma volume as well as an increase in hematocrit. This occurs in response to stressful stimuli. It is known that catecholamines cause both an increase in blood pressure and an increase in venous tone and resistance. Therefore, in response to stress, fluid is forced from the blood into interstitial spaces and the plasma volume drops. This increase in blood viscosity is what contributes to the formation of plaques on the arterial walls as well as the aforementioned cardiovascular diseases.

Personality type is thought to play a role in CHD as well. People with a type A personality are prone to competitiveness, hostility and anger. It is these traits that are major risk factors in CHD because they would induce greater stress in the individual than a more relaxed personality (Manuck *et al.* 1988). These type A individuals demonstrate more hemodynamic properties when stressed and are therefore at more risk for CHD. The sympathetic nervous system is thought to play a role in CHD because it is observed in monkeys that dominant individuals have an increase in atherosclerosis which can be inhibited by β-adrenergic blockers (Manuck *et al.* 1988).

In addition to acute psychological stress, psychosocial factors such as depression, work-related stress (job strain) and chronic stress have all been linked with an increased risk of atherosclerosis (Rozanski *et al.* 1999). Depression is known to induce hypercortisolemia due to increased activation of the HPA. Depression is associated with impairments in platelet function, which produce proatherogenic effects.

Platelets are now thought to be critical in the development of atherosclerosis (Brydon *et al.* 2006). Atherosclerosis is a chronic inflammatory process involving leukocytes and endothelium of the arteries (Ross 1999). Platelets facilitate the interaction between leukocytes and the artery wall. When platelets are activated, P-selectin is increased on their surface (Merten *et al.* 2004). P-selectin binds to P-selectin glycoprotein ligand-1 (PSGL-1) which facilitates both an interaction with leukocytes to form platelet-leukocyte aggregates (PLAs) as well as the rolling of platelets and leukocytes on the endothelium of the arteries (Merten *et al.* 2004; Brydon *et al.* 2006). The recruitment of leukocytes to the endothelium results in an increased lesion size. It is believed that dysfunctional endothelial cells, inflammation and the recruitment of leukocytes all contribute to the formation of lesions (Huo *et al.* 2003). In addition to the changes to P-selectin, activated platelets also release an increased amount of adhesive and pro-inflammatory factors to the leukocytes and endothelial cells (Huo *et al.* 2004). These factors include chemoattractants to recruit leukocytes, monocytes, fibroblasts and vascular smooth muscle cells to the endothelial wall (Huo *et al.* 2004; Brydon *et al.* 2006). These cells are stimulated to proliferate in the lesions. Monocytes in these lesions produce inflammatory cytokines and free radicals. Activated platelets also express CD40 ligand (CD40L), which binds to CD40 on the endothelial cells and upregulates chemokines and adhesion molecules further recruiting leukocytes to the site (Brydon *et al.* 2006). This whole process contributes to the progression of atherosclerosis. Stress and psychosocial factors are thought to participate in the progression of atherosclerosis by activating platelets. PLA formation increases and these complexes last longer in the circulation after stress (Brydon *et al.* 2006). The

exact mechanisms of the effects of stress on PLA formation are unclear but it has been observed that catecholamines can stimulate the activation of platelets (von Kanel *et al.* 2000; Steptoe *et al.* 2003; Brydon *et al.* 2006); however there are studies to the contrary. The evidence that acute stressors affect atherosclerosis is inconsistent and further research is required (Steptoe *et al.* 2003).

2.4.2 Insulin Resistance and Type-2 Diabetes

Diabetes mellitus type 2 (T2D) is a disease characterized by insulin resistance (IR) and hyperglycemia. Insulin is the protein responsible for the regulation of glucose uptake and metabolism. Insulin resistance is a disorder where the individual's cells have a decreased sensitivity to insulin. This decreased sensitivity leads to a decreased ability of insulin to activate metabolism of glucose, increased plasma concentrations of insulin, dyslipidemia (decreased plasma triglycerides and decreased high density lipoprotein cholesterol) and hyperglycemia, which is a large increase in circulating glucose (Black 2006). Stress has been implicated as one of many contributing factors to IR and T2D (Pliquett *et al.* 2004; Black 2006). It has been observed that catecholamines can induce a decreased activation of the insulin receptor (Pliquett *et al.* 2004; Black 2006). This is very obvious in the case of pheochromocytoma patients. This disorder induces high catecholamine production and very low sensitivity to insulin (Pliquett *et al.* 2004). One mechanism by which stress can act to induce insulin resistance is by inhibiting insulin signaling molecules such as insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2) (Klein *et al.* 1999). Insulin binding to its receptor activates a signaling cascade inside the cell (Klein *et al.* 1999). The receptor induces

phosphorylation of several substrates including IRS-1 and IRS-2. These substrates then interact with Src homology-2 (SH-2) domain containing protein phosphatidylinositol 3-kinase (PI-3 kinase). PI-3 kinase leads to the activation of Akt and stimulates active glucose uptake (Klein *et al.* 1999). It is by SNS activation (β-adrenergic stimulation) that this cascade is inhibited. Phosphorylation of IRS-1 and to a lesser extent IRS-2 is decreased and PI-3 kinase activity is suppressed therefore, glucose uptake is diminished and insulin sensitivity of the cell is decreased (Klein *et al.* 1999). Insulin can also activate the SNS creating a positive feedback loop which further increases IR (Pliquett *et al.* 2004). There is an ongoing epidemic of diabetes mellitus type 2 in North America and along with obesity, inactivity and hypertension; stress is contributing to the onset of this disease.

2.4.3 Rheumatoid Arthritis and Juvenile Idiopathic Arthritis

Rheumatoid arthritis (RA) is a chronic autoinflammatory disorder which affects the joints. The immune system attacks the joints causing pain and decreased mobility. RA is most commonly found in the elderly whereas juvenile idiopathic arthritis (JIA) is found in young people. Since arthritis is an inflammatory disease, it is not surprising that stress can influence it. In this case, stress can act both as a disease-permissive factor and a disease aggravator (Straub *et al.* 2005). It is well known that acute, minor stressors have an immunostimulatory effect whereas major, chronic stressors have an immunosuppressive effect (Mawdsley *et al.* 2005; Straub *et al.* 2005). At low cortisol concentrations, like those seen after stimulation with an acute stressor, production of IL-6 and TNFα by macrophages is increased in response to lipopolysaccharide (Barber *et*

al. 1993; Mawdsley *et al.* 2005; Straub *et al.* 2005). This type of stressor stimulates a pro-inflammatory response and thus may contribute to the onset of arthritis by acting as a disease-permissive factor (Straub *et al.* 2005). This enhancement of inflammation within the correct autoimmune environment allows for the accommodation of this disease.

Stress can also play the role of disease aggravator. Once symptomatic arthritis appears, stress can contribute to the severity (Straub *et al.* 2005). In the presence of certain stress axes defects, stress-induced aggravation can occur. These defects include inadequate secretion of cortisol after stimulus, inadequate sympathetic tone after stimulus and a shift at the site of arthritis from β-adrenoceptors to α-adrenoceptors on certain immune cells such as macrophages, neutrophils and NK cells. These defects can all contribute to a lack of immunosuppression and create an environment which promotes inflammation and aggravates arthritic disease (Straub *et al.* 2005).

2.4.4 Mood Disorders/Depression and Learning Impairment

Stress has been indicated as a potential contributing factor to mood disorders and depression. It has been observed that during depression, the levels of a number of nerve growth factors are decreased in an area of the brain called the hippocampus (Duman *et al.* 2006). The hippocampus is part of the limbic system and is involved in learning as well as memory storage and retrieval (Fenoglio *et al.* 2006). The nerve growth factors involved in depression are brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT-3) (Duman *et al.* 2006). These all play a role in the survival and functioning of neurons in the CNS. During depression, the levels of all

three factors are decreased in the hippocampus. It is hypothesized that the inhibition of neurogenesis, due to insufficient quantities of nerve growth factors is the origin and cause of depression and associated behavioral symptoms (Duman *et al.* 2006). Stress has been shown to exert the same effects on BDNF, NGF and NT-3 levels (Smith *et al.* 1995; Ueyama *et al.* 1997; Duman *et al.* 2006). In regards to BDNF, the effects of many types of stressors have been studied. Forced swim stress, social isolation and immobilization stress are examples of what has been studied and each one has had the same effect: they have all decreased the amount of BDNF in the hippocampus (Smith *et al.* 1995; Barrientos *et al.* 2003; Roceri *et al.* 2004). This change can be mediated by cortisol because when animals are administered corticosterone, BDNF is decreased just as with stress (Smith *et al.* 1995; Schaaf *et al.* 1998). BDNF mRNA increases after adrenalectomy also suggesting that cortisol is responsible for the stress-induced decrease (Chao *et al.* 1998). However, removal of the adrenal glands does not completely block the effects of stress on BDNF signifying that there could be other contributing factors (Duman *et al.* 2006). Nonetheless, there is considerable evidence to suggest that stress could play a role in the development of depression.

The depression hypothesis involves stress which is experienced during adulthood. Severe early life stress or stress in early childhood has much different consequences. Severe stress early in the development of a child leads to impaired cognitive function including learning disabilities and memory problems later in life (Fenoglio *et al.* 2006). As is often the case, acute mild stress has an enhancing effect on the hippocampus and its learning and memory processes whereas severe or chronic stress has the opposite effect (Luine *et al.* 1996; Fenoglio *et al.* 2006). The CA3 area of

the hippocampus is especially affected by early life stress because it develops late compared to other structures in the brain. In early post-natal hippocampal development, expression of CRH receptors (CRF_1) is significantly higher than levels found in the adult hippocampus (Baram *et al.* 1998; Fenoglio *et al.* 2006). For this reason, when the child is stressed early in life and CRH levels increase in the brain, the CA3 neurons of the hippocampus are damaged due to excitotoxicity. High levels of CRH damage neurons expressing CRF_1 (Baram *et al.* 1998). CRH is also chronically up-regulated by early severe stress so that future stress responses will cause a greater CRH response (Fenoglio *et al.* 2006). All of these early stress effects are supported by the observation that CRH-overexpressing mice have learning and memory impairment compared to normal mice and post-natal administration of CRH causes the same effects as severe early stress (Fenoglio *et al.* 2006). Stress has a profound effect on the hippocampus. Mild stress can act to enhance hippocampal function but chronic or severe stress has detrimental effects which impair function.

2.5 Immunological Effects

As early as 1964, a link was known to exist between stress and immunity (Solomon *et al.* 1964). Since then the effects of stress on immune function have been widely studied (Borysenko *et al.* 1982; Yang *et al.* 2000; Padgett *et al.* 2003). It is important to study this link because it often leads to susceptibility to disease as in the case of bovine respiratory disease (BRD) in cattle (Galyean *et al.* 1999). BRD can be caused by a primary infection with a virus, commonly bovine herpesvirus-1 (BHV-1), followed by a secondary bacterial infection with *Mannheimia haemolytica* (Lillie 1974).

BRD causes huge economic losses to the cattle industry every year through the loss of animals as well as treatment costs. Stressors such as transportation have been associated with susceptibility to BRD. Therefore, it is very important to understand the effects of stressors on the immune system.

It is known that cortisol levels increase after a stressful event due to the activation of the HPA. To elucidate the mechanisms of stress-induced immune modulation, studies to determine the effects of cortisol on certain parameters of the immune system were carried out (Borysenko *et al.* 1982; Blalock 1989; Yang *et al.* 2000). It has been observed that cortisol can inhibit the function of macrophages, basophils, mast cells, neutrophils and eosinophils (Borysenko *et al.* 1982). Glucocorticoids and ACTH have been shown to affect the proliferation of B- and T-cells, cytokine production, antibody production, chemotaxis of monocytes and neutrophils and NK cell cytotoxicity (Blalock 1989). Many of the effects of glucocorticoids occur at low concentrations within the range that can be induced by stress (Borysenko *et al.* 1982).

Activation of the SAM axis also occurs after stress resulting in increased levels of epinephrine and norepinephrine. It is known that lymphocytes have β-adrenergic receptors and when these receptors are stimulated by catecholamines adenylate cyclase is activated and cAMP is produced (Borysenko *et al.* 1982; Yang *et al.* 2000). Increases of cAMP in lymphocytes increase metabolism of immature lymphocytes, which causes maturation of these cells. However, cAMP inhibits mature lymphocytes (Borysenko *et al.* 1982; Yang *et al.* 2000). Elevation of cAMP has also been shown to decrease histamine release by human basophils and decrease antibody production to sheep red

blood cells in mice (Borysenko *et al.* 1982; Yang *et al.* 2000). One important observation to note is that in vitro treatment of peripheral blood leukocytes (PBLs) with catecholamines results in the inhibition of IL-12 synthesis and an increase in IL-10 production (Elenkov *et al.* 1996; Yang *et al.* 2000; Padgett *et al.* 2003). This shift in cytokine production causes a shift in T-helper (Th) cells from Th1 cells to Th2 cells. Th1 cells are involved in cell-mediated immunity whereas Th2 cells are involved in antibody production (Yang *et al.* 2000). A shift away from a Th1 cell mediated response can result in increased susceptibility to viral infections (Elenkov *et al.* 1996). The same shift towards a Th2 type response and away from a Th1 type response was seen in healthy medical students taking exams (Marshall *et al.* 1998). A decrease in interferon-γ (IFN- γ) and an increase in IL-10 from isolated PBMCs were observed after exam stress. Exam stress was also shown to decrease NK activity, decrease PBL's responses to mitogen, decrease production of IFN- γ by stimulated PBLs, and decrease antibody and virus-specific T-cell response to a hepatitis B vaccine (Glaser *et al.* 1985; Glaser *et al.* 1987; Glaser *et al.* 1992; Yang *et al.* 2000). An increased rate of upper respiratory tract infection was also reported (Yang *et al.* 2000). Another study took healthy individuals and inoculated them with five strains of live respiratory viruses (Cohen *et al.* 1991; Yang *et al.* 2000). They found that those with psychological stress had an increased risk for developing respiratory illness. Also, the higher the stress the more severe the symptoms (Cohen *et al.* 1991).

In addition to studying the effects of cortisol and catcholamines on the immune system, some studies employ dexamethasone (DEX) to study the effects of stress on cattle (Pruett *et al.* 1987; Anderson *et al.* 1999). Dexamethasone is a synthetic

glucocorticoid. It has commonly been used to simulate stress-induced immunosuppression (Pruett *et al.* 1987). Studies using DEX have found a decrease in antibody production as well as suppression of bovine lymphocyte proliferation (Ohmann *et al.* 1987; Pruett *et al.* 1987). These results concur with the effects of glucocorticoids and catecholamines.

Both the HPA and SAM axes have been shown to influence many aspects of the immune system. Activation of these leads in most cases to reduced immunity and increased susceptibility to infection. Bovine respiratory disease is a perfect example of how stress can lead to increased disease rates.

2.6 Stress Indicators

In order to properly study stressors and their effects, a valid and reliable indicator of stress must be determined. Some attempts have been made to find reliable markers of stress. Cortisol levels, catecholamine levels, acute phase proteins and even behavioral changes have all been used as ways to evaluate the stress of an individual. However, none of these indicators are without problems.

2.6.1 Cortisol

Since stress is known to induce the HPA and cortisol is the end product of that cascade, it is reasonable to assume that cortisol levels can be used to indicate stress. An increase in cortisol levels has long been used to indicate stress (Beerda *et al.* 1996; Grandin 1997; Queyras *et al.* 2004). Cortisol levels can be monitored in saliva, serum and urine (King *et al.* 2002). The advantages of using cortisol as a marker include its

non-invasive measurement and direct connection to the stress response of the body. It also has several disadvantages (Queyras *et al.* 2004). Cortisol is naturally secreted in a diurnal rhythm. This makes it important to consider the time of day you are sampling. Cortisol naturally spikes in the morning and reaches a low around midnight (Queyras *et al.* 2004). This diurnal cycle adds some complexity to using cortisol as a marker. Another problem with using cortisol as a marker, is that not all types of stressors induce an increase in cortisol. Weaning in cattle is an example (Hickey *et al.* 2003). Clearly for these stressors another marker is needed for their identification. Studies have also shown that there is significant inter- and intra-individual variability in cortisol levels suggesting that when using cortisol levels, each individual should serve as its own control (Queyras *et al.* 2004). In addition, due to the negative feedback cortisol has on components of the HPA, cortisol levels can not be used to gauge long-term stressors. High cortisol levels can take no longer than 90 minutes to return to baseline levels (Queyras *et al.* 2004). Increases in cortisol levels also occur very rapidly after stress. Taking blood from an animal results in an increase in cortisol levels in as little as two minutes (Queyras *et al.* 2004). Cortisol increases are a good indicator of stress; however, the many disadvantages limit its use to specific studies. Perhaps a marker with fewer disadvantages can be found.

2.6.2 Catecholamines

If cortisol is used as a marker because it is an end product of HPA induction then catecholamines can be used as well since they are the result of SAM activation. Many stress studies include the quantification of epinephrine and norepinephrine (Lefcourt *et*

al. 1995; Loerch *et al.* 1999; Hickey *et al.* 2003). However, catecholamines are rapidly eliminated from blood (Hjemdahl 1993). They have also been shown to have a diurnal variation thus studies must be standardized for time of sample collection (Hjemdahl 1993). Plasma catecholamines represent the overall sympathetic activity and caution is necessary when using them to assess a condition since the organs most contributing to plasma levels might not be involved in the studied condition (Hjemdahl 1993). Any condition activating the sympathetic nervous system would show an increase in catecholamines. Therefore, they are not a specific marker of stress.

2.6.3 Behavioral Changes

Behavior of animals can be used as an indicator of stress (Queyras *et al.* 2004). Studies have shown weaning in cattle causes a predictable change in behavior such as increased vocalizations, more walking and less time eating and resting (Veissier *et al.* 1989; Haley *et al.* 2005). Branding and isolation among other stressors are known to cause an increase in vocalizations in cattle (Watts *et al.* 2000; Watts *et al.* 2001). These changes in behavior can be used to identify stressed animals, however, not all stressors induce a change in behavior (Queyras *et al.* 2004). Behavior is also not specific to a certain type of stressor; therefore additional physiological measures may be needed to reliably identify the effects of each type of stressor.

Proteomics methodologies have sufficient resolution and sensitivity to identify candidate biomarkers for stress such as weaning stress. Serum is a good sample candidate because it can represent the overall changes in physiology due to weaning and also it is easily obtainable. Selection of the current experimental methodology was also

dictated by the fact that it should identify the indicators while introducing minimal artefacts and requiring no drastic treatment of the sample. Finally it was decided to study the global profile of water soluble proteins in serum extracted from the blood of experimental animals. The methodology of studying the whole protein profile of a system is referred to as proteomics.

2.7 Proteomics Methodology

2.7.1 The Use of Two-dimensional Gel Electrophoresis in Biomarker Discovery

Proteomic methodologies are at the forefront of biomarker discovery (Bischoff *et al.* 2004; Thadikkaran *et al.* 2005). Since changes at the transcription level do not necessarily correlate with changes in expression of proteins, studying the proteome gives a better picture of what is happening functionally in the individual (Thadikkaran *et al.* 2005). Proteomics techniques make it easier to identify changes or differences between a treatment or disease and a control. One such proteomic technique is two-dimensional gel electrophoresis (2D-GE). Serum, as well as other bodily fluids, can be analysed using this technique so less invasive sample collection is possible (Young *et al.* 1995). Its advantages include using a mixed protein sample as well as having a high resolving power (Young *et al.* 1995; Bischoff *et al.* 2004). Staining of proteins in-gel using Coomassie G-250 stain has a detection limit of 8-10 ng (Miller *et al.* 2006). Many studies have successfully used 2D-GE to discover novel biomarkers (Celis *et al.* 1999; Celis *et al.* 2000; Seliger *et al.* 2002). Lately, the focus has shifted from finding a single biomarker to identify a disease or condition to identification of a pattern of markers. 2D-GE allows for the discovery of a pattern of protein changes because, with this

technique, all proteins in the samples are analysed together (Young *et al.* 1995; Bischoff *et al.* 2004). A pattern rather than a single protein allows for a more stringent identification with less false positives (Bischoff *et al.* 2004).

2D-GE in combination with mass spectrometry (MS) facilitates the discovery of markers. Improvements to MS techniques in the 1990s made rapid identification of separated proteins possible by comparing sequence data with database information (Steen *et al.* 2004; Thadikkaran *et al.* 2005). Unlike surface enhanced laser desorption/ionization mass spectrometry (SELDI-MS) analysis, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) allows for the identification of separated proteins. SELDI-MS can rapidly detect changes or differences between samples but does not provide sequence information for identification (Thadikkaran *et al.* 2005).

2.7.2 Limitations

Bischoff and Luider (2004) note several limitations of the 2D-GE technique. With 2D-GE, it is difficult to display the entire proteome. Often proteins which have a low solubility or extremely acidic/alkaline isoelectric points will not appear on the gel. As well, proteins larger than 200 kDa and smaller than 10 kDa will not be displayed on the gel. When analyzing serum samples, additional limitations exist. Albumin is very abundant in serum and therefore is usually removed from the samples before analysis in human studies (Thadikkaran *et al.* 2005). However, with studies involving cattle and other animals where the serum albumin is slightly different, standardized kits for the removal of human serum albumin are not optimized for the removal of bovine serum

albumin. Some studies suggest that the presence of high abundance proteins such as albumin creates a bias towards high abundance proteins and diminishes detection of medium and low abundance proteins (Gygi *et al.* 2000). This is a major limitation of 2D-GE.

MS characterization also has a major limitation. The identification of the proteins of interest depends on the completeness of the protein databases used. This becomes an issue when studying the bovine proteome where the protein database is no where near as complete as the human protein database (D'Ambrosio *et al.* 2005). This does not limit the discovery of differences but merely limits the identification of the proteins involved. Despite these limitations, 2D-GE and MS analysis are still valuable tools in the discovery of biomarkers as well as the study of various proteomes (Thadikkaran *et al.* 2005).

3.0 HYPOTHESIS AND OBJECTIVES

Stress has profound effects on humans and animals alike. There are numerous examples of stressors identified for cattle. These induce stress responses in the animal including the induction of the HPA axis and the SAM system. They have varying detrimental effects on the welfare and health of cattle and have been shown to contribute to the susceptibility of diseases such as bovine respiratory disease. Weaning stress is one component of a series of stressors which a calf encounters during its lifetime. It is important to study each of these stressors and their effects in order to better prevent disease susceptibility and improve animal welfare. Weaning is an example of a stressor which does not show an increase in cortisol levels. An increase in cortisol levels is a known indicator of stress. Since weaning stress cannot be identified using cortisol, another marker must be discovered. A pattern of protein changes which are unique to weaning stress would make an excellent identifier. To find these unique protein changes, proteomics techniques such as 2D-GE and MS were employed.

We **hypothesize** that weaning will cause sufficient stress in cattle to alter protein profiles in serum, which can then be used to identify this type of stress.

The objectives of this study were:

1) To determine the effects of abrupt weaning on serum proteins by comparing an abrupt weaned group with a group that had never been weaned.
2) To determine whether pre-conditioned animals have a protein profile similar to control animals, which have never been weaned
3) To determine the effects of pre-conditioning by comparing this group to animals who have undergone abrupt weaning.
4) To determine the effects of abrupt weaning on serum proteins over the course of the trial.

4.0 MATERIALS AND METHODS

4.1 Clinical Parameters

Body weight and temperature measurements were carried out on all calves each time they were handled. Body weight was measured in kilograms using a chute scale (Senstec 3000U). Each calf was run through the cattle chute individually and confined on the scale while a measurement was taken. Temperature was measured with a handheld, M500 digital rectal thermometer (GLA Electronics; CA, USA).

4.2 Behavioral Observations

Four calves from each group were randomly chosen for behavioral observations. Observations were made on experimental days 1, 2 and 3 post-weaning. Each animal was observed over a 10 minute period by one of two observers. At the beginning of each minute, the animal's behavior was noted. The observer recorded which of several categories best described the animal's behavior. The available categories were: lying (L), lying while ruminating (LR), standing (S), standing while ruminating (SR), eating (Eat), walking (W), drinking (D) and, in the case of never weaned calves only, nursing (N). During each minute the number of vocalizations (VOX) made by each calf was also recorded. These observations were then compared among the treatment groups.

4.3 Serum Isolation from Blood

Approximately ten milliliters of whole blood was collected from each calf in serum-separator tubes (SST) (BD Vacutainer; NJ, USA) by venipuncture. Samples were collected at approximately 10 AM, however due to the number of animals being processed, the collection time varied slightly. Samples were also collected from animals in a random order each collection day. Blood samples were allowed to stand at room temperature for one hour. Serum was separated by centrifugation at 1350 x g for 10 minutes. Serum was collected in a clean Eppendorf tube and Protease Inhibitor Cocktail (Sigma-Aldrich; ON, Canada) was added (100 µl / 1 ml of serum). Each sample was then separated into 100 µl aliquots and stored at -80˚C.

4.4 Cortisol Concentration Assay

Cortisol concentration was determined by Prairie Diagnostic Services for each serum sample using the Immulite system (Diagnostic Products Corporation; CA, USA).

4.5 Quantification and Isoelectric Focusing

Quantification of the protein in each serum sample was done using the Bradford protein assay (Bradford 1976). Bovine serum albumin was used as a standard. A 1:500 dilution was made of each sample. Twenty microlitres of diluted sample was added to 1 ml of Bradford dye (Cat #500-0001, Bio-Rad laboratories; PA, USA), vortexed and incubated for 5 minutes. Absorbance was measured using a Lambda 25 UV/VIS spectrometer (Perkin Elmer Instruments; Quebec, Canada) at a wavelength of 595 nm.

Two milligrams of serum protein was mixed with rehydration buffer [4 % (w/v) Chaps, 48% (w/v) urea, 1% Pharmalyte pH 3-10 (GE Healthcare; Quebec, Canada), 0.2% (w/v) dithiothreitol and a trace of bromophenol blue in ddH_2O] for a final volume of 250 µl and applied to 13 cm Immobiline DryStrips pH 3-10 (GE Healthcare; Quebec, Canada) which were allowed to rehydrate overnight. Once rehydrated, isoelectric focusing was run in three steps which were: 500 V for one hour, 1000 V for one hour and finally 8000 V for two hours using the Ettan IPGphor II IEF system (GE Healthcare; Quebec, Canada). For all three steps, current was set at 50 µA per strip at 20 ˚C. After IEF, the strips were equilibrated with dithiothreitol (GE Healthcare; Quebec, Canada) in equilibration buffer [36% (w/v) urea, 3.35% 1.5M tris pH 8.8, 34.5% glycerol, 2% sodium dodecyl sulfate and a trace of bromophenol blue in ddH_2O] followed by iodoacetamide (GE Healthcare; Quebec, Canada) in equilibration buffer.

4.6 SDS-PAGE

Each strip is then placed on top of a 12% polyacrylamide gel (12% acrylamide/bisacrylamide solution, 25% 1.5M tris pH8.8, 0.1% sodium dodecyl sulfate, 43.4% ddH_2O, 0.05% ammonium persulfate and 0.1% temed) and run at 25 volts per gel for approximately 4 hours. The gels were then fixed in a fixing solution (50% ethanol, 2% orthophosphoric acid and 48% ddH_2O) overnight followed by staining with Coomassie G250 stain [Bio-Rad (PA, USA); 34% Methanol, 17% (w/v) ammonium sulfate, 2% orthophosphoric acid, 0.1% (w/v) coomassie G250 and 62% ddH_2O] for 4 hours and destaining in 25% methanol for approximately 30 mins. Gels were scanned using an Epson Expression 1680 scanner. Images were saved as 16-bit tiff images.

4.7 Mass Spectrometry

Excised protein spots were destained in 25% methanol until the gel fragment was clear. The protein samples were then treated with a reducing solution (10 mM dithiothreitol and 100 mM ammonium bicarbonate) followed by a blocking solution (50 mM iodoacetamide and 100 mM ammonium bicarbonate). Protein samples were digested overnight using TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone) treated trypsin. Resulting peptides were extracted three times into a 96-well plate. Peptide mass fingerprinting analysis of each sample was carried out on an Applied Biosystems Voyager DE-STR (Foster City, CA) equipped with a nitrogen laser operated at 337 nm with a 3 ns pulse. The instrument was operated in positive ion reflectron mode. The surface of the stainless steel MALDI plate was made hydrophobic using a variation of the method reported by Owen *et al* (2003). The surface of the MALDI plate was sprayed with a commercially available hydrophobic spray (FootLocker Water and Stain Repellent; Foot Locker Canada, Weston ON) and allowing to dry for 15 mins. This was repeated four times and allowed to dry overnight. To the prepared plate, 1.5 µl of α-cyano-4-hydoxycinnamic acid (CHCA) matrix (5 mg/ ml in 75% acetonitrile and 0.1% trifluoro-acetic acid) and 5 µl of protein digest was added to a spot on the plate and mixed. The spots were then allowed to dry. Four hundred laser shots were averaged for each spot and then smoothed, background corrected and converted to the monoisotopic values with Data Explorer (Applied Biosystems; CA, USA). The spectra were internally calibrated with the autolytic fragment from trypsin (MH^+ 842.5100 m/z) when present and when not present a close external calibration was done using a mixture

of des-Arg1 Bradykinan (MH^+ 904.4681 m/z) and ACTH (18-39) (MH^+ 2465.1989 m/z). The resulting peak lists were submitted to Protein Prospector (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) for database searching.

4.8 Data Analysis

Intensity values were obtained for each protein spot. These values were called percent volumes. The software determines the volume of each spot which is a measure of each spot's intensity. It then adds up all the spot volumes on one gel creating a total volume value for each gel. Each spot volume was then divided by the total volume for that gel. The result is a percent volume value for each spot on each gel. A percent volume value is used for analysis because it is a way of normalizing the data. Using a percent volume value enables a cross gel comparison to be made. Each gel was run separately and stained independently. Even when controlling for variables like staining time, differences in staining darkness may still exist. For this reason, normalized data was used to make comparisons among groups or treatment days.

Statistical analysis was used to determine whether there were any statistically significant differences in the data for each comparison. The data had a parametric trend; therefore, a t-test was used to determine if the treatment groups were different and a one-way ANOVA with a Tukey post hoc test was applied to determine which day was significantly different. A one-way ANOVA was also used to detect changes over time within each group. A statistically significant difference among the treatment groups or for the same treatment group over the days of the trial was one with a p-value of less

than or equal to 0.05. Potential markers of weaning stress would be protein spots which were statistically different between the abrupt weaned group and the control group.

5.0 EXPERIMENTAL DESIGN

5.1 Trial 1: Method Validation

Two dimensional gel electrophoresis and mass spectrometry were selected as the methodologies of choice to address the hypothesis which states that weaning will cause sufficient stress to alter the serum protein profile in calves. In order to optimize and determine whether changes in protein levels could be observed using these techniques, a small animal trial was planned. DEX was used as an artificial means of stressing the calves. The purpose of this trial was to apply a stressful condition and determine if we could observe any changes when compared to a control. From this trial we obtained bovine serum samples which were analyzed using the two techniques. The experimental design is outlined in Table 5.1.

On the first day of the trial (Day 0) blood was collected by venipuncture from three four month old calves. This day 0 sample was our control sample. The calves had not been treated with DEX at this point. After the control sample was collected, the animals were injected intramuscularly with 10 mg of DEX. Starting on day 1, blood was collected daily from each calf. Serum was isolated from all blood samples collected during this trial. The samples from one calf were used for optimization of the aforementioned two-dimensional gel electrophoresis protocol.

Table 5.1 Experimental timeline for Dexamethasone-treated calves

Day 0 (Control)	Day 1	Day 2	Day 3
• Collect blood in SST tubes for serum • Inject 10 mg DEX/animal	• Collect blood in SST tubes for serum	• Collect blood in SST tubes for serum	• Collect blood in SST tubes for serum

5.2 Trial 2: Weaning stress

To test our hypothesis, a weaning stress trial was planned. Thirty cow/calf pairs were randomly selected from a herd. All pairs selected were moved into one large pen three weeks before the trial began where they remained for the entire trial. The three week adaptation period was used to eliminate or reduce the effects of other types of stressors such as social reorganization and change in the environment. Cow/calf pairs were randomly allocated to three treatment groups: an abrupt weaned group (AW), a never weaned group (NW) and a preconditioned group (PC). Calves in the NW group remained with their dams throughout the entire period of the trial. This group was an absolute control because they never underwent weaning. All samples from this group reveal the state of calves prior to weaning. Cows from the PC group were separated from their calves and the cows were removed from the pen three weeks prior to beginning the trial. This was to ensure that these calves underwent weaning stress and then recovered prior to initiating the weaning stress trial. This is an accepted type of weaning stress control (Hodgson *et al.* 2005). Finally, the AW group consists of calves which were weaned at the beginning of the trial. This group was undergoing weaning stress throughout the trial period. Figure 5.1 is a timeline for the trial and shows when each of the experimental manipulations was carried out.

From Figure 5.1, all animals including the calves and their cows were moved into a large pen away from the rest of the herd on day -21. Blood for serum was collected from all calves at this time. Animals were given one week to acclimatize to their new surroundings and peers. Social reorganization and changes in the environment

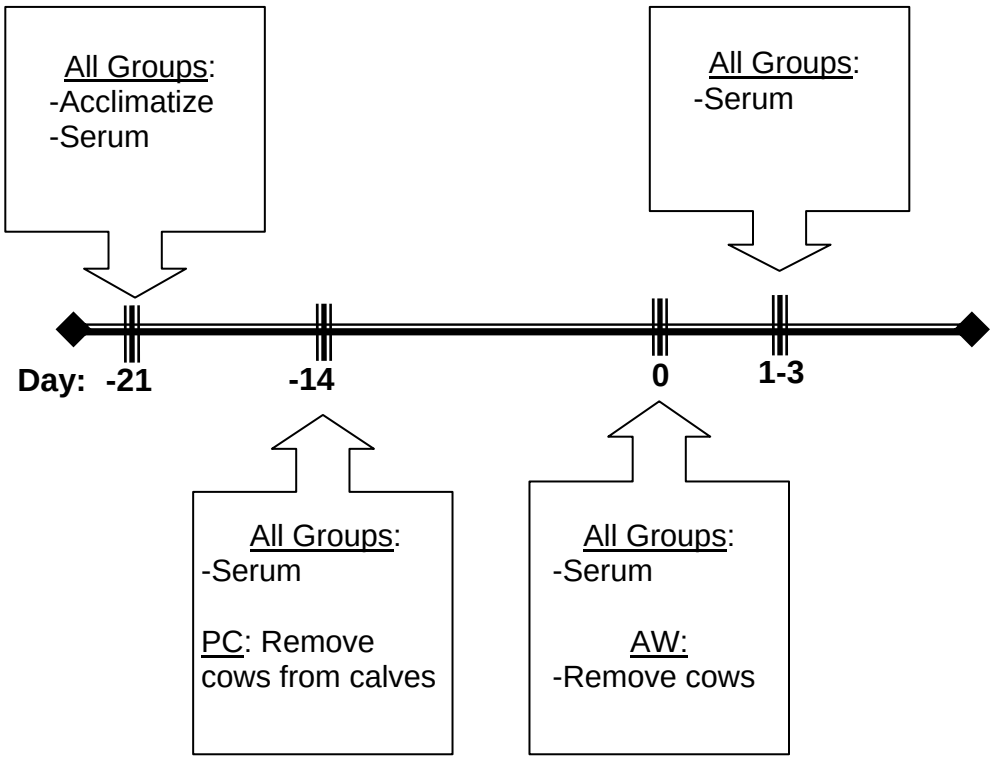


Figure 5.1: Weaning stress trial timeline. Days are denoted along the timeline. Each text box describes what was done on each day and to which treatment groups.

are well known psychological stressors. In this trial, we wished to study only weaning stress. Therefore, it was very important to eliminate as many other stressors as possible. On day -14, serum was collected from all calves and the dams of the preconditioned group were removed from the pen to wean the calves in this group. Note that the dams were removed from the pen but not the calves. All experimental animals remained in the same environment throughout the entire trial. PC calves were allowed two weeks to recover from being weaned. Up until day 0, all events were part of a preparatory phase. Day 0 marked the beginning of the experimental phase. On day 0, serum was collected from all calves and the cows of the AW calves were removed from the pen. The calves of the AW group were now experiencing weaning stress and would do so for the remainder of the trial period. Day 0 serum samples were considered to be control samples. On this day, the AW group and the NW group were in the same state since weaning had not taken place for AW. The PC group had been previously weaned and was now in a state after weaning stress had taken place. On days 1 through 3, serum was collected daily from all calves at approximately the same time. These samples, including day 0, comprised the samples for protein analysis from this trial.

In addition to serum, several behavioral and clinical observations were made daily when the calves were handled. These measurements included body weight and temperature, which were used to monitor the calves for effects on weight gain or loss due to stress as well as fever or illness. In the event that one or more of the calves developed a body temperature greater than 40.2˚C for at least two consecutive days, they would have been removed from the study and treated accordingly. However, this never occurred. Behavioral observations were made several hours after handling on

days 1, 2 and 3. Four calves were selected randomly from each treatment group to be observed for behavior which included time spent lying, standing, eating, walking and drinking. These behavioral observations are ones typically studied with respect to weaning stress. It has been shown that there is a statistically significant difference between the behavior of calves prevented from nursing and those which are allowed to nurse (Haley *et al.* 2005). All cow/calf pairs in this study were kept on rangeland pasture.

Six animals from each group were selected for proteomic analysis due to time and cost constraints. Six animals from each group give sufficient power to achieve necessary statistical significance. Serum collected in this trial went through a series of steps in analysis. As shown in Figure 5.2, the serum was isolated from the blood samples and first analysed for cortisol concentration. All samples then underwent two-dimensional gel electrophoresis and scanned gel images were obtained. These gel images were then analysed using an image analysis software (Image Master Platinum, GE Healthcare Biosciences, Quebec, Canada) where normalized intensity values for each protein were calculated. Comparisons between each treatment group and within each treatment group over the days of the trial were made using the normalized intensity values. Analyses were performed to determine whether any changes observed across groups or within groups but between days of the trial were statistically significant. It was determined by frequency distribution analysis that the intensity data had an approximately normal distribution therefore a one-way analysis of varience (ANOVA) was used. The gels from 2D-GE were also used for protein identification. Protein spots were selected and excised from the gel and used for mass spectrometric analysis. The

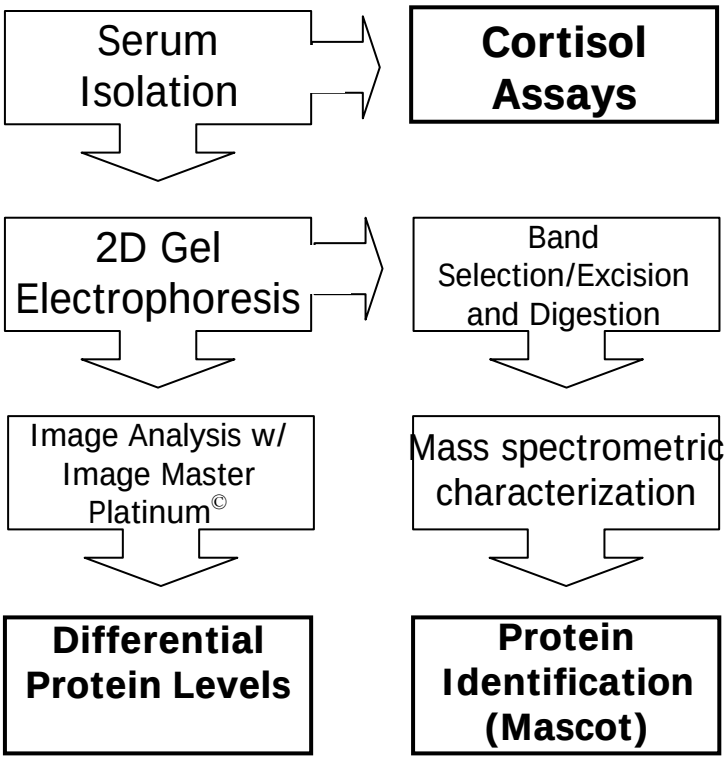


Figure 5.2: Analysis flowchart for serum samples. Serum was first isolated from the blood samples and used for cortisol assays to determine their cortisol concentration. Next two-dimensional gel electrophoresis was carried out using the serum to obtain gel images for analysis using Image master platinum software as well as mass spectrometric analysis. Differential protein levels were obtained by comparing intensity levels calculated during image analysis. Protein identification was obtained by using mass spectrometry data to search a protein database. Mascot from Matrix Science was the search engine used.

resulting peak lists from each digested protein were compared against all protein databases using Mascot search engine from Matrix Science (http://www.matrixscience.com/) and protein identifications were made.

6.0 RESULTS

6.1 Trial 1: Method Validation

6.1.1 Gel Images

Sera collected from one calf on each of the four experimental days were used for two-dimensional gel electrophoresis. Figure 6.1 shows representative gel images for each day of the trial. The same two areas of each image are boxed to highlight examples of areas on the gels where changes in protein levels were detected over the course of the trial. In box A of the control sample (day 0), there were no detectable protein spots. After injection of DEX, some protein spots appear on day 1 in box A. On the second day after injection, more protein spots are visible in addition to those present on day 1. The trend continues on day 3, where even more protein spots were detected. In box B, a different trend was apparent. On day 0 there were no measurable protein spots but after injection, spots appear on day 1 and 2 but were no longer detectable on day 3. By looking at the gel images, these qualitative observations can be made. Quantitative comparisons can also be done using image analysis software.

6.1.2 Quantitative Analysis

Normalized intensity values obtained from the scanned gel images were used to compare protein spots on each day of this trial. Two areas of the gels were found to show changes over the course of the trial. Figure 6.2 reports the intensity of each spot in box A as a percent volume value. When comparing the values for each spot over the

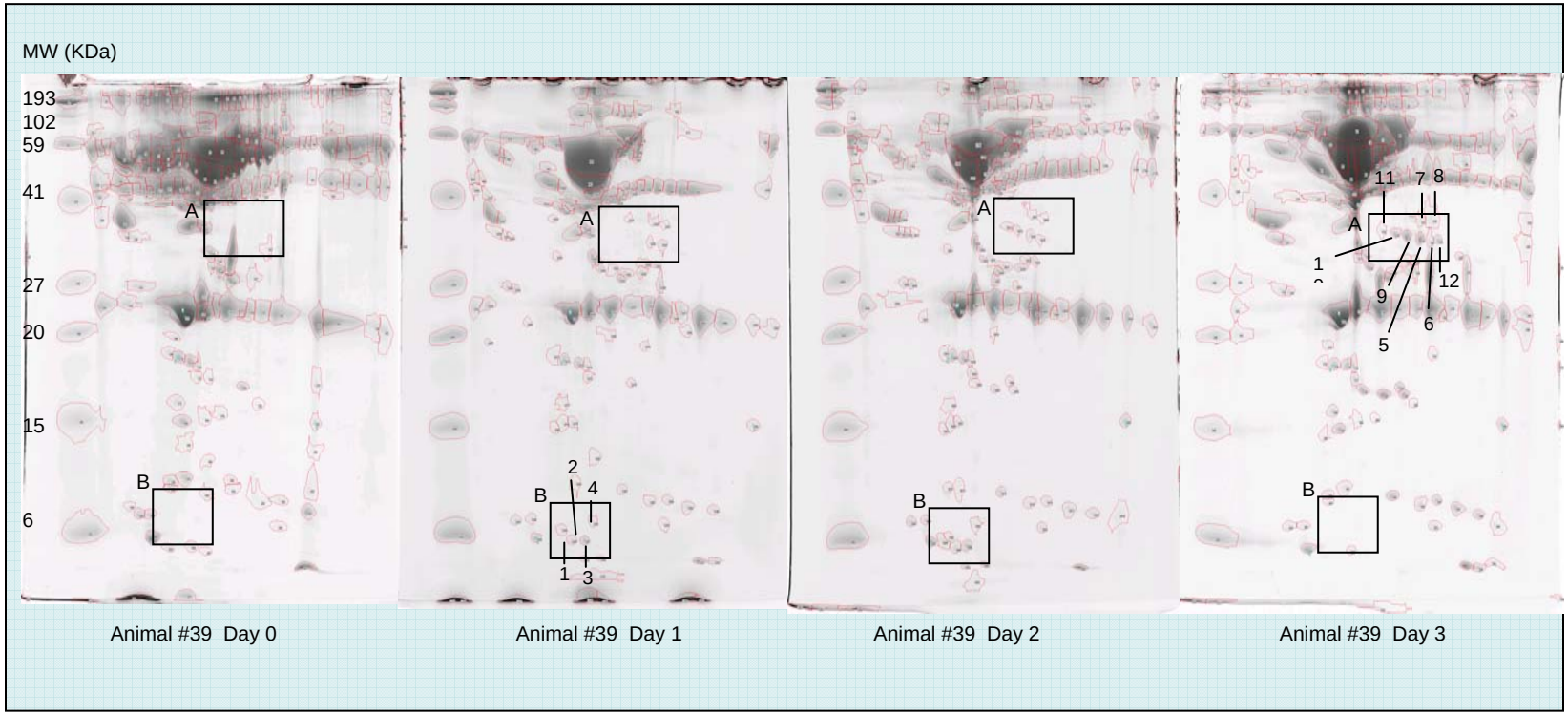


Figure 6.1: Gel images from animal #39 on each day of the trial. Day 0 is a control sample taken before dexamethasone injection. Days 1,2 and 3 are samples on each day following dexamethasone injection. Boxes A and B are examples of areas in which a detectable difference in protein levels was observed. Individual protein spots in each box were assigned a number.

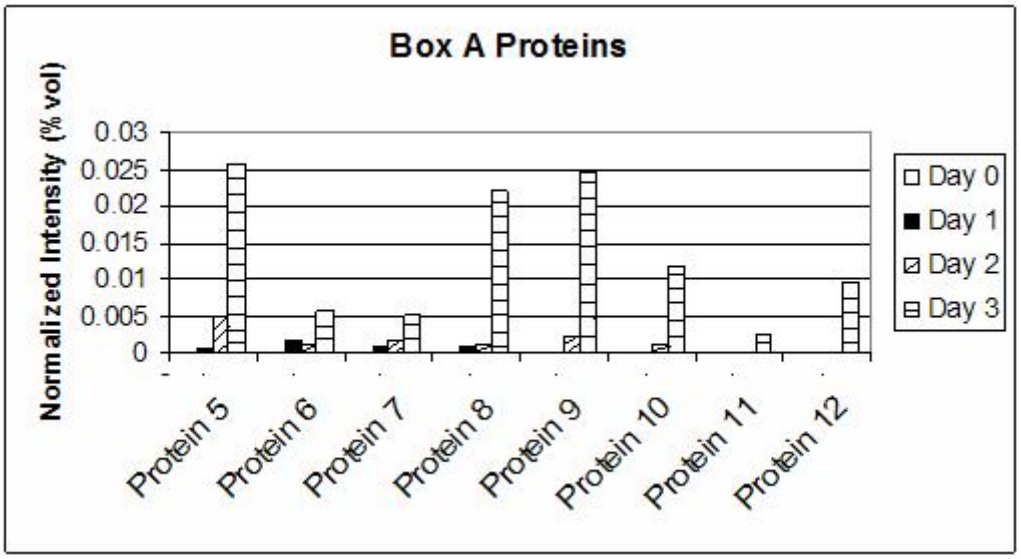


Figure 6.2: Box A protein intensities from Figure 6.1. Normalized intensity (y-axis) is over protein number (x-axis). Each day is represented by a single bar. Days 0 through 3 are shown for each spot in box A.

days of the trial, the trend was similar for all eight spots. On day 0, the value of each box A protein was zero, meaning these spots were not detectable on that day. The values of each spot gradually increase with each day to reach a maximum value on day 3.

In Figure 6.3, the four box B proteins are presented. All proteins have an intensity value of zero on day 0, meaning they were not detectable on that day. After injection of dexamethasone, all four proteins appear and increase in intensity from day 1 to day 2. On day 3 all four proteins have returned to an intensity of zero.

As mentioned earlier, box A and box B are representative examples of where changes were observed over the days of this trial. A discussion of this trial and these results will follow in the discussion section.

6.2 Trial 2: Weaning Stress

6.2.1 Body Weight and Temperature

The body weight of each calf in each treatment group was recorded on the days when samples were collected (days 0, 1, 2 and 3). This was done to monitor the body weight gain or loss of each calf. Figure 6.4 reports the body weights of each calf over the course of the trial. There was no statistically significant body weight gain or loss in any calf irrespective of the treatment group. There was also no statistically significant difference in body weight gain or loss when compared among treatment groups. All calves remained at approximately the same weight throughout the course of the trial.

Body temperature was also measured and recorded for each calf on days that samples were collected. Monitoring animals for fever and illness was very important

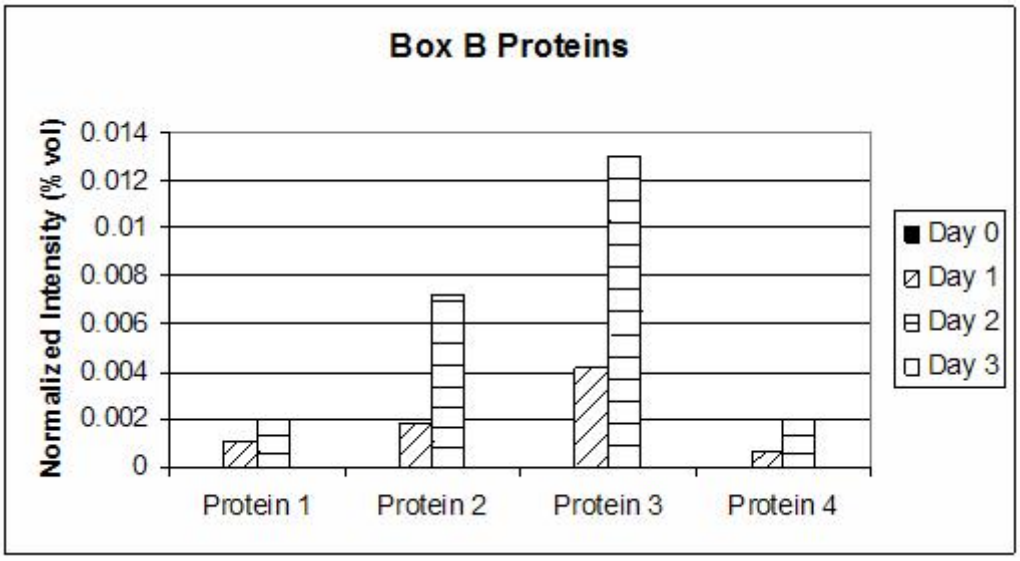


Figure 6.3: Box B protein intensities from Figure 6.1. Normalized intensity (y-axis) is over protein number (x-axis). Each day from day 0 to day 3 is represented by a bar. All protein intensities on day 0 are zero and gradually increase until day 2 where they peak and return back to zero on day 3.

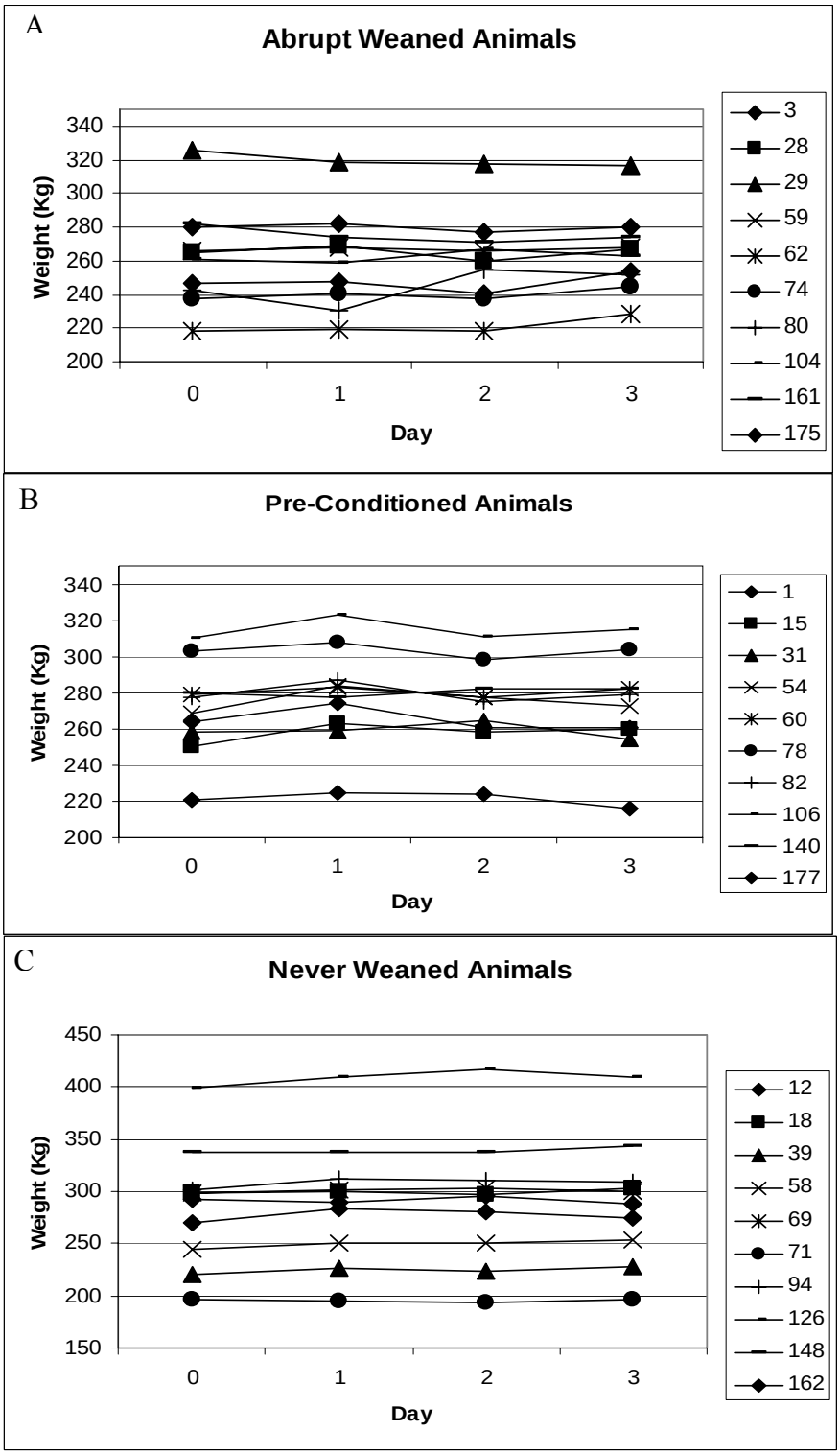


Figure 6.4: Body weight in kilograms of each calf in A) the abrupt weaned group, B) the never weaned group and C) the preconditioned group over the days of the trial (0, 1, 2 and 3). Animal numbers are shown to the right of the graph. Each line joining points shows the body weight trend for each animal over the course of the trial.

since illness is a stressor. Had any calves developed a fever of greater than 40.2˚C for two consecutive days, they would have been removed from the trial. None of the calves developed a fever and all remained in the study (Figure 6.5). Animal #1 from the PC group had a transient increase in body temperature on day 2 (40.2˚C) and on day 3 (40.1˚C).

6.2.2 Behavioral Observations

Four calves were randomly chosen as representative animals from each treatment group and were observed for a period of ten minutes on days 1, 2 and 3. Figure 6.6 presents the recorded observations as an average value for all four animals in each group. The y-axis represents the number of times each behavior was observed. It is the mean of four animals. Statistical analysis of the data revealed no statistical differences among the treatment groups or on individual days within treatment groups. However, the AW group was the only group to make vocalizations. From this data, overall it was not possible to differentiate between the groups.

6.2.3 Serum Cortisol

Samples collected at approximately the same time each day from all thirty animals were analyzed for serum cortisol concentration. As mentioned earlier, in the past an increased level of cortisol has been used as a marker for stressed individuals. In Figure 6.7, the cortisol concentrations for individual animals in each group are shown for days 0, 1, 2 and 3. In the AW group, there was no significant increase in cortisol levels after the calves were weaned. On days 1, 2 and 3, the serum cortisol levels were similar when compared with the control (day 0). There were also no differences among

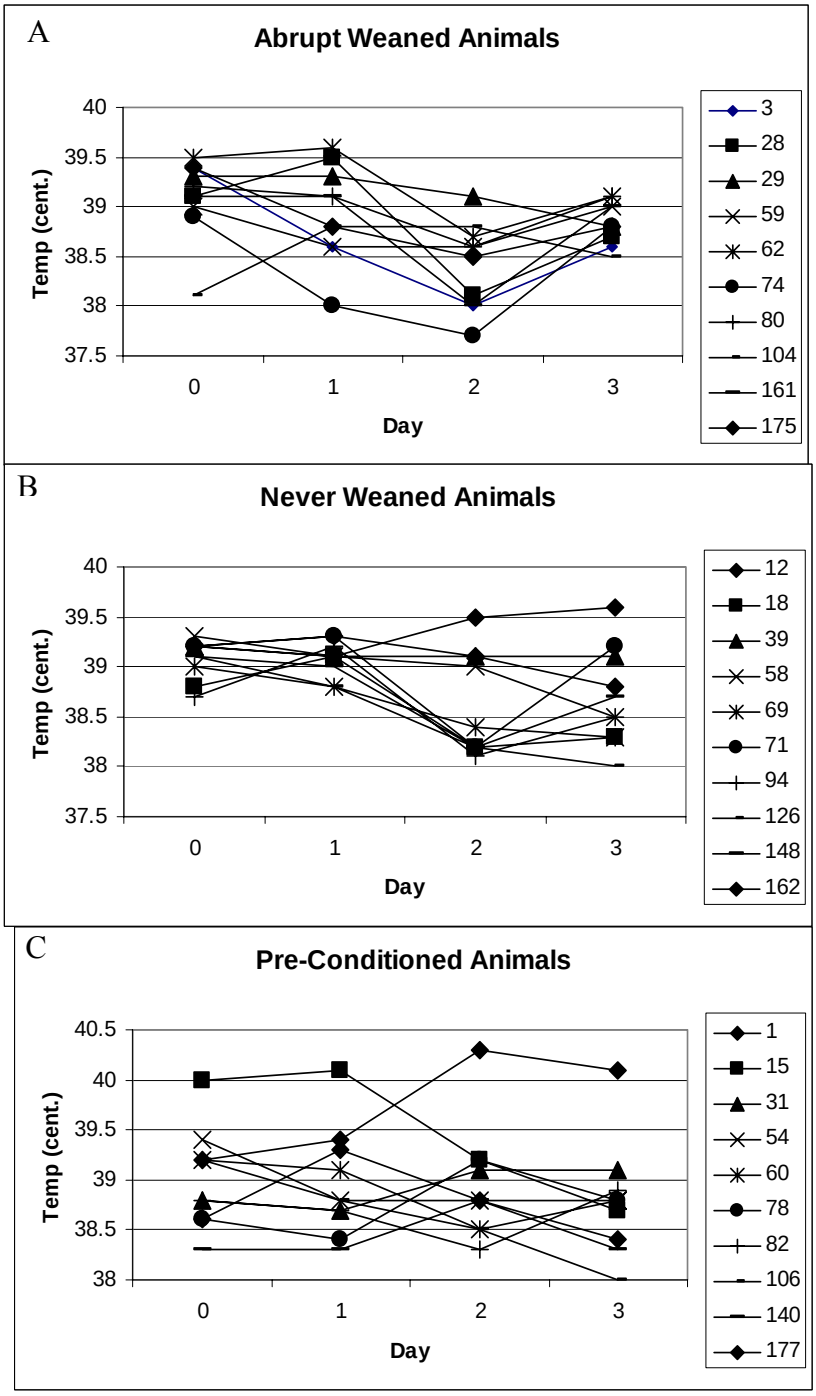


Figure 6.5: Body temperature in degrees Celsius for each calf in A) the abrupt weaned group, B) the never weaned group and C) the preconditioned group over the days of the trial (0, 1, 2 and 3). Animal numbers are shown to the right of the graph. Each line joining points shows the temperature changes for each animal.

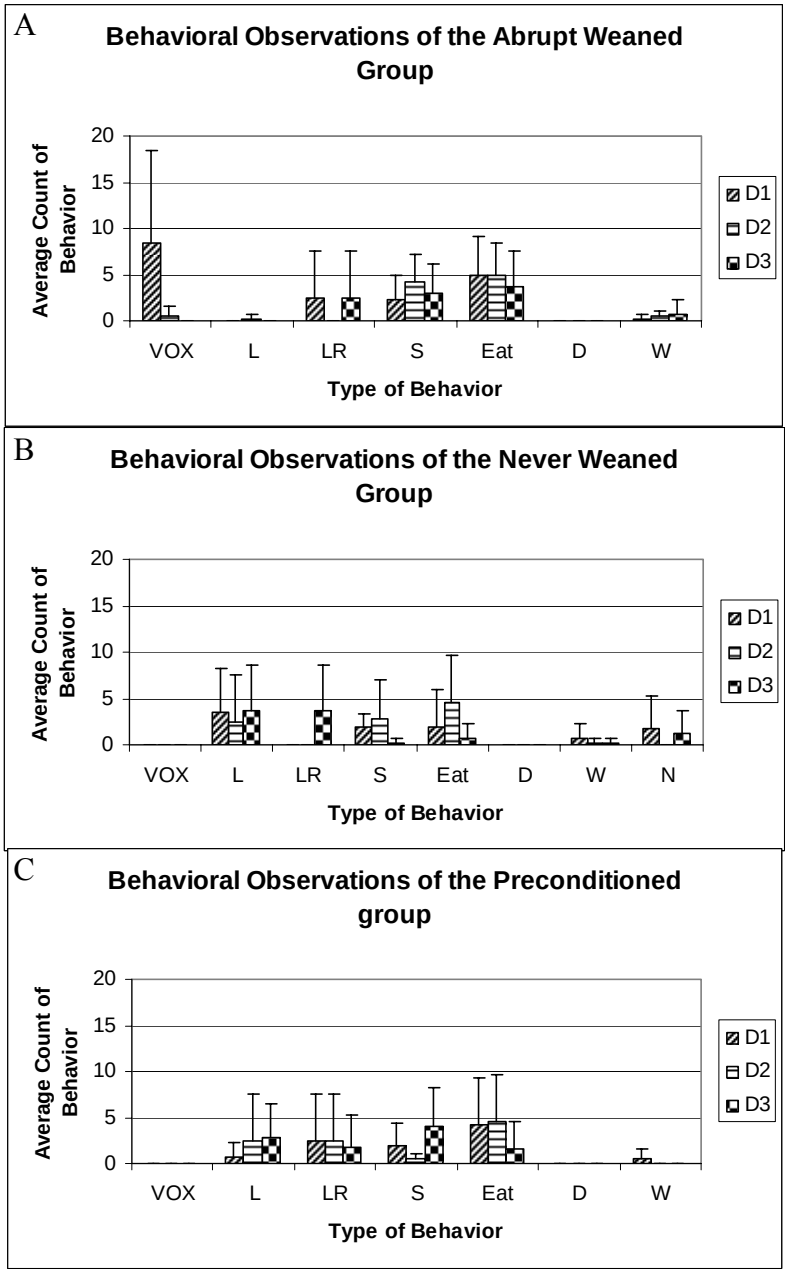


Figure 6.6: Behavioral observations of each treatment group. Types of behaviors monitored included VOX-vocalizations, L-lying, LR-lying while ruminating, S-standing, SR-standing while ruminating, Eat-eating, D-drinking and W-walking. For the never weaned group N-nursing was added. Behavioral observations of A) the abrupt weaned group, B) the never weaned group and C) the preconditioned group are shown. Type of behavior is along the x-axis while the average count of each behavior is along the y-axis. The results for all four animals in each treatment group were averaged. Days 1, 2 and 3 are shown. Day 1 is the first day after weaning of the abrupt weaned group. SR had counts of 0 for each group on each day.

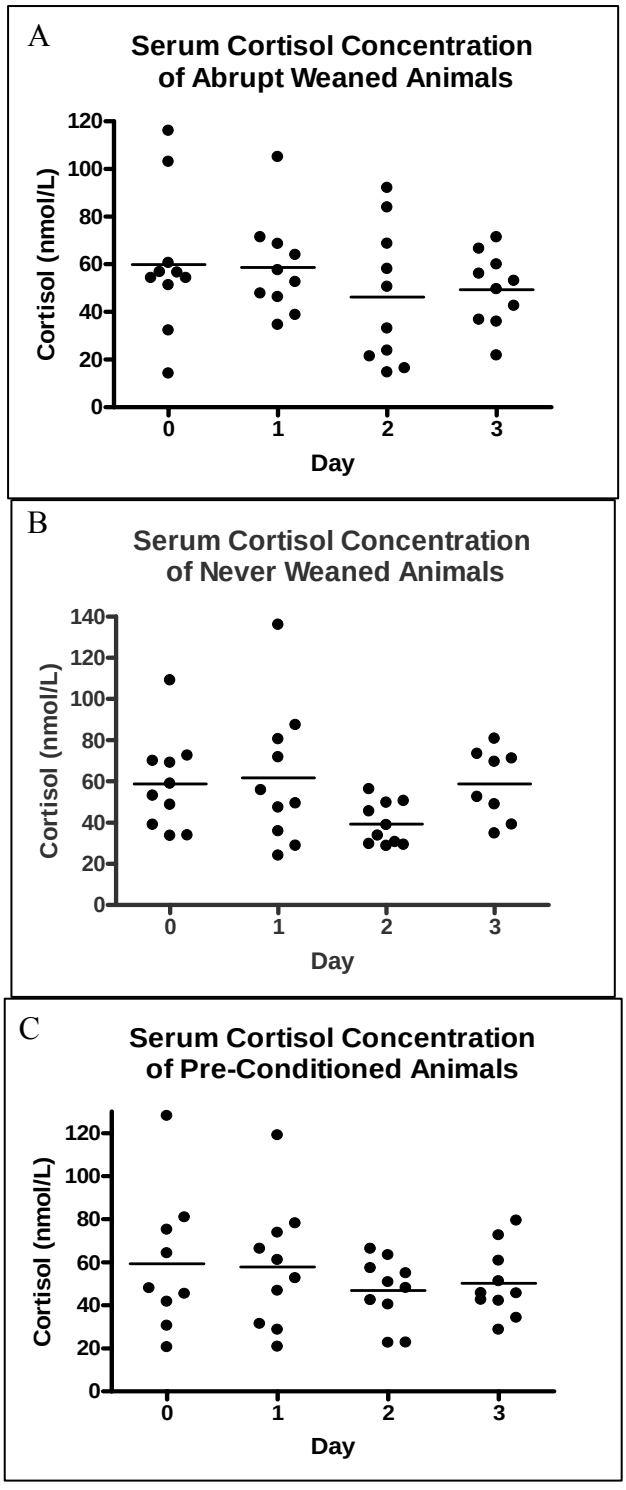


Figure 6.7: Serum cortisol concentrations in each treatment group on days 0, 1, 2 and 3. Cortisol concentrations in nmol/L for A) the abrupt weaned group, B) the never weaned group and C) the preconditioned group are presented for individual animals. The mean serum cortisol concentration for each group is denoted by a horizontal bar.

the experimental days (days 1, 2 and 3) and the control in either the NW group or the PC group. In addition, there were no significant differences in the serum cortisol levels when comparing among treatment groups. Throughout the trial, the serum cortisol levels remained relatively constant over time and across groups. Six animals from each treatment group were selected for two-dimensional analysis on the basis of the cortisol data. These six animals from each group best represented the median cortisol values in each group overall. Animals which had slightly different cortisol values than the median were not chosen.

6.2.4 Gel Images

Two-dimensional gel electrophoresis was performed on sera samples from the six animals on days 0 through 3. Day 0 is the control day and days 1 through 3 are the experimental days after the AW calves have been weaned. One gel image from each treatment group on day 0 is shown (Figure 6.8) with the AW group represented by animal 104 (Panel A) the NW group is represented by animal 18 (Panel B) and the PC group represented by animal 78 (Panel C). Boxes A, B and C are areas in which statistically significant differences were found when protein intensities were compared among treatment groups. These boxed areas have been magnified to show greater detail in Figures 6.9, 6.10 and 6.11.

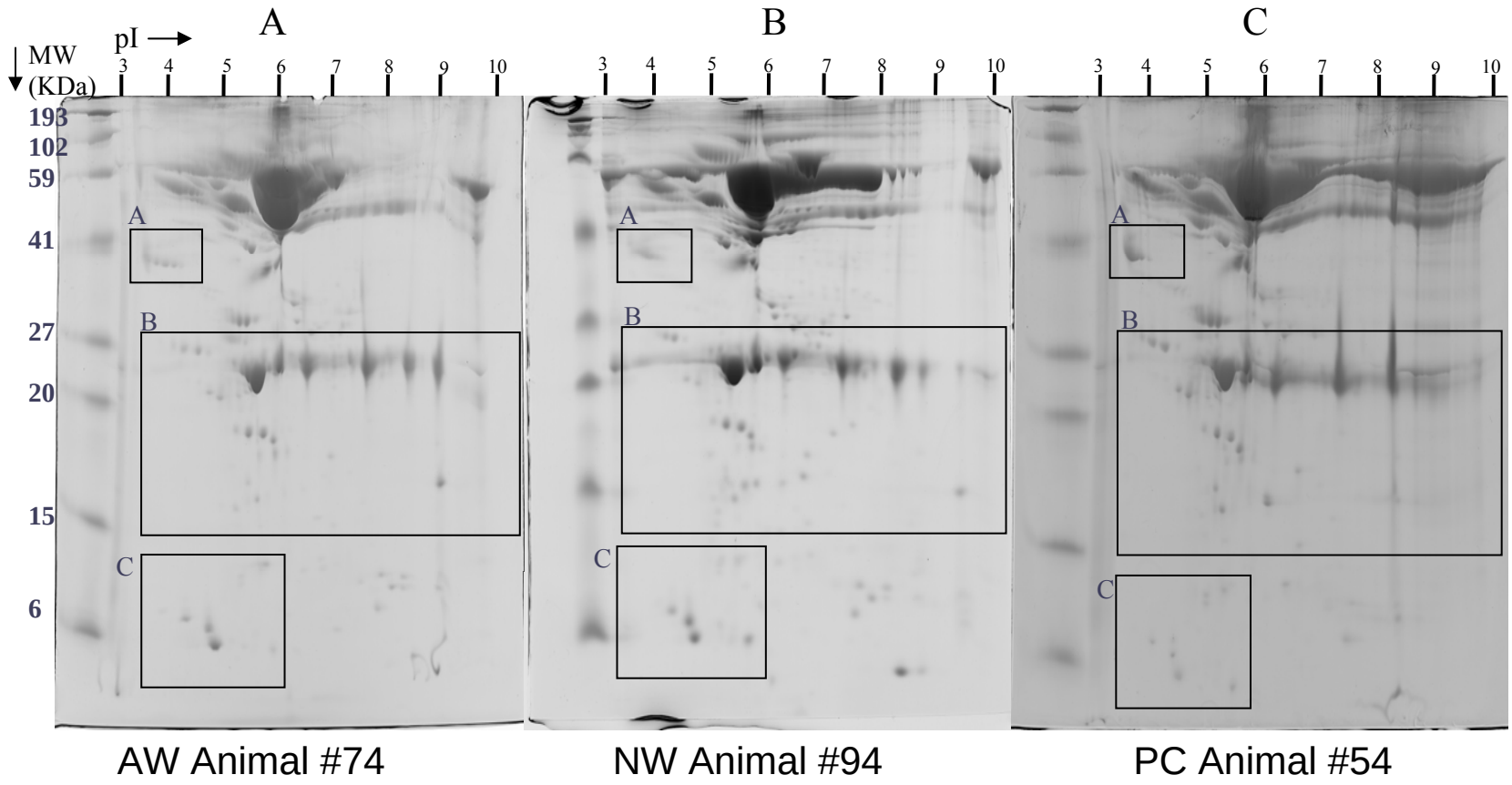


Figure 6.8: Representative 2D-GE images of serum proteins for each treatment group: A) Abrupt weaning group represented by animal # 74, B) Never weaned group represented by animal #94 and C) Pre-conditioned group represented by animal #54. All images depict day 0. Boxes A, B and C are areas in the gel images where statistically significant differences were identified when proteins were compared among treatment groups.

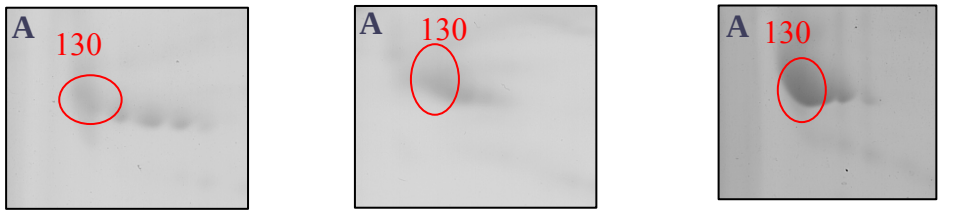


Figure 6.9: Magnified region A from the three gels in Figure 6.8. Spot 130 is highlighted in this box because it was statistically different among treatment groups. On the left is box A from animal #74 of the AW group. In the center is box A from animal #94 of the NW group. All images depict day 0. On the right is box A from animal #54 of the PC group.

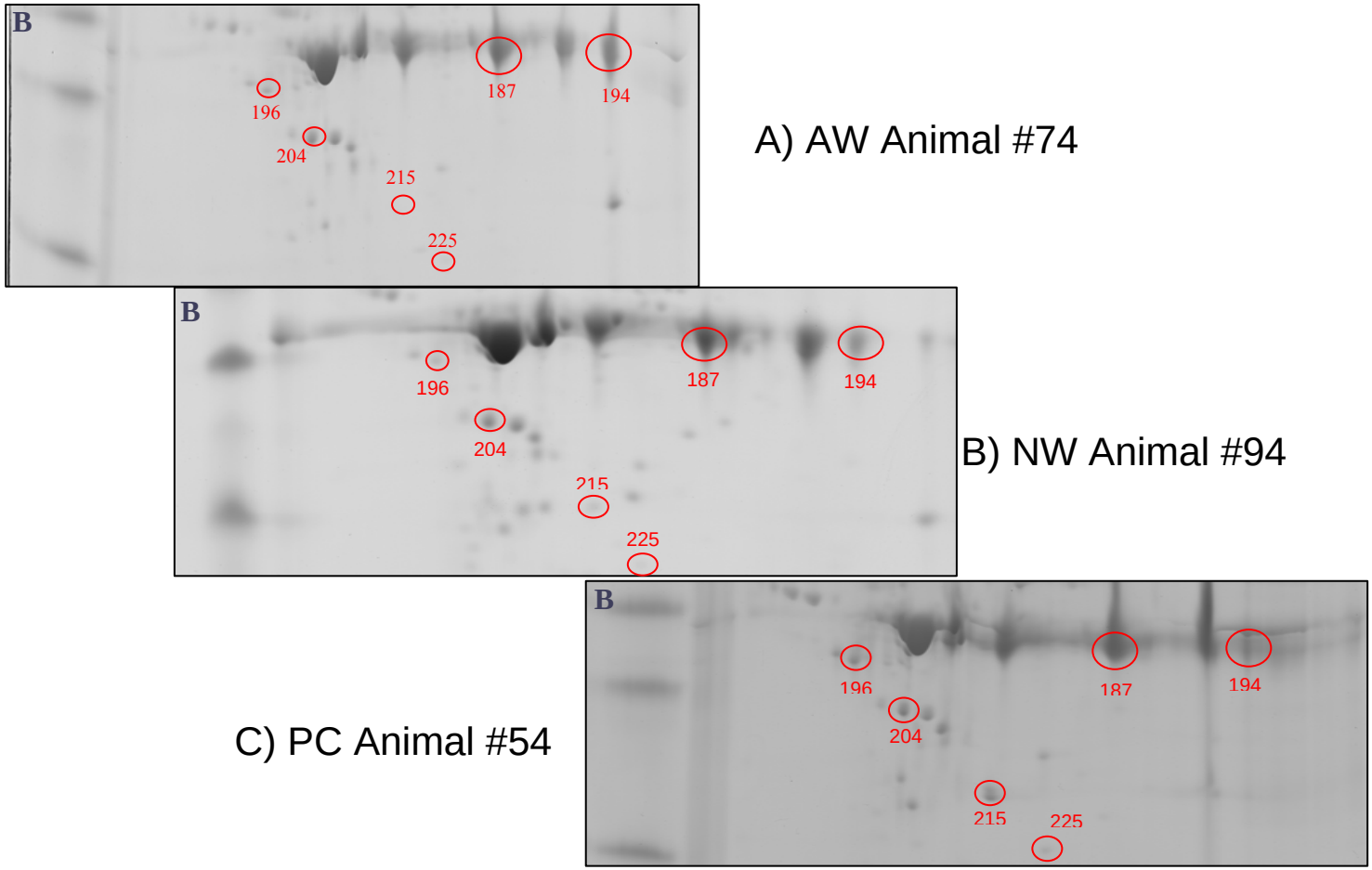


Figure 6.10: Magnified region B from the three gels in Figure 6.8. All highlighted spots were statistically different in intensity when compared among the three treatment groups. Box B of A) animal #74 of the AW group, B) animal # 94 of the NW group and C) animal #54 of the PC group is shown. All images depict day 0.

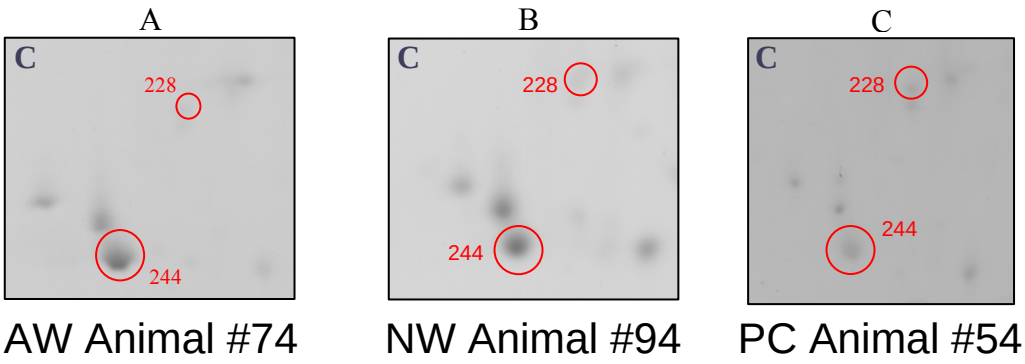


Figure 6.11: Magnified region C from each gel in Figure 6.8. Highlighted spots are those which were statistically different in intensity when compared among the three treatment groups. A) box C from animal #74 of the AW group B) box C from animal #94 of the NW group and C) box C from animal #54 of the PC group are shown. All images depict day 0.

6.2.5 Significant Proteins

Two different types of comparisons were made using the normalized intensity values obtained from the scanned gel images. Protein intensities were compared among the three treatment groups and within groups over the course of the trial. Statistical methodologies have been applied to the intensity data in order to determine if there were statistically significant differences in either of these two comparisons. As expected since the animals were selected from an outbred population, the animal to animal variation was high. A one-way ANOVA takes into account both the variability within each group and among the groups. In total, nine protein spots were found to be statistically different when either of three following comparisons were made; the AW group was compared with the NW group, the AW group was compared with the PC group or the NW group was compared with the PC group. Seventeen protein spots were found to change significantly over the course of the trial when comparing within groups.

When comparing the protein intensities of the AW group to those of the NW group, two protein spots were found to be significantly different when using a one-way ANOVA. The results of such analysis are outlined in Figure 6.12. Any difference with a p-value of less than 0.05 was considered significant. Spot 187 was significantly different in intensity on day 1 with intensity greater in the AW group than in the NW group. Day 1 was the first day after the AW group was weaned. There were no significant differences for this spot on days 2 and 3; therefore, this difference between the two treatment groups was very transient. Spot 215 also differed on days 1 and 3, however the difference in intensity on day 3 was not unique to the AW group. The effects on the serum proteome of weaning stress seem to only persist for one day

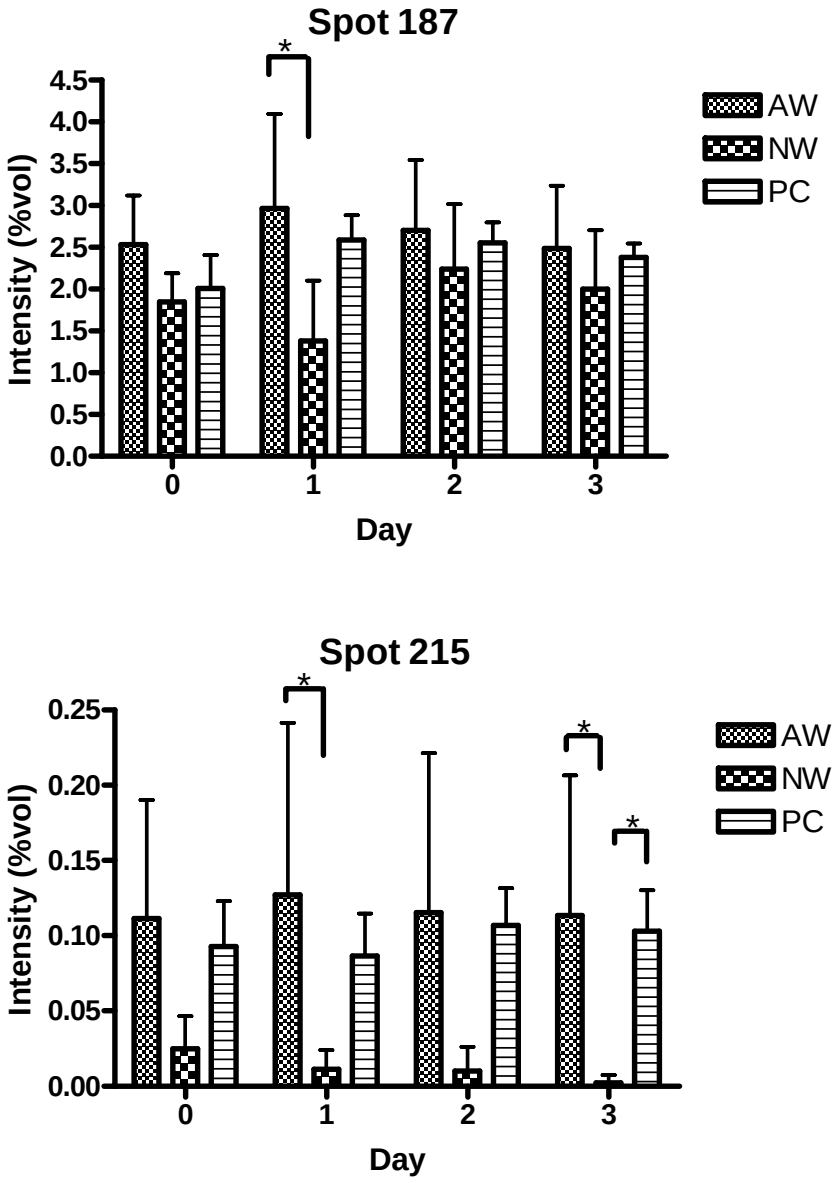


Figure 6.12: Comparison of spot intensities for the AW and NW group. Each graph represents the intensities of one protein spot. Bars marked with * have a p-value < 0.05.

after weaning. The transient nature of this protein change would not provide a good biomarker for weaning stress responses since that was an issue with cortisol as well.

Five proteins were significantly different in intensities when the AW group and the PC group were compared (Figure 6.13). Spot 130 showed a difference in intensity on both day 0 and day 1. Spot 194 was significantly different on day 0 and spots 196 and 228 were significantly different in intensities only on day 2. Lastly, spot 244 was significantly greater in intensity in the AW group than in the PC group on day 1. These differences between the AW and PC groups were distributed throughout the time course including differences on day 0. Since the PC group was weaned much earlier than the AW group, differences on day 0 might be expected.

Figure 6.14 shows five proteins which were significantly different in intensity when the PC group was compared with the NW group. Spot 194 was different only on day 1. Spots 204 and 225 both were different on day 2; however, spot 225 was also different on day 0. Spots 196 and 215 were different between the two groups on day 3. Again, all these differences in protein intensities had a very transient nature. This analysis showed that the serum proteome of these two control groups, PC and NW, were different. Therefore, depending on what physiological parameters are studied, the choice of control group is very important and may change the results of the study.

Statistical analysis was also performed in order to identify protein spots which may be changing over the course of the trial within each group. A total of seventeen proteins were found to change significantly over time when analyzed within group with a one-way ANOVA. A p-value of less than 0.05 is considered significant. Two spots from the NW group changed significantly over the course of the trial. Spot 209 differed

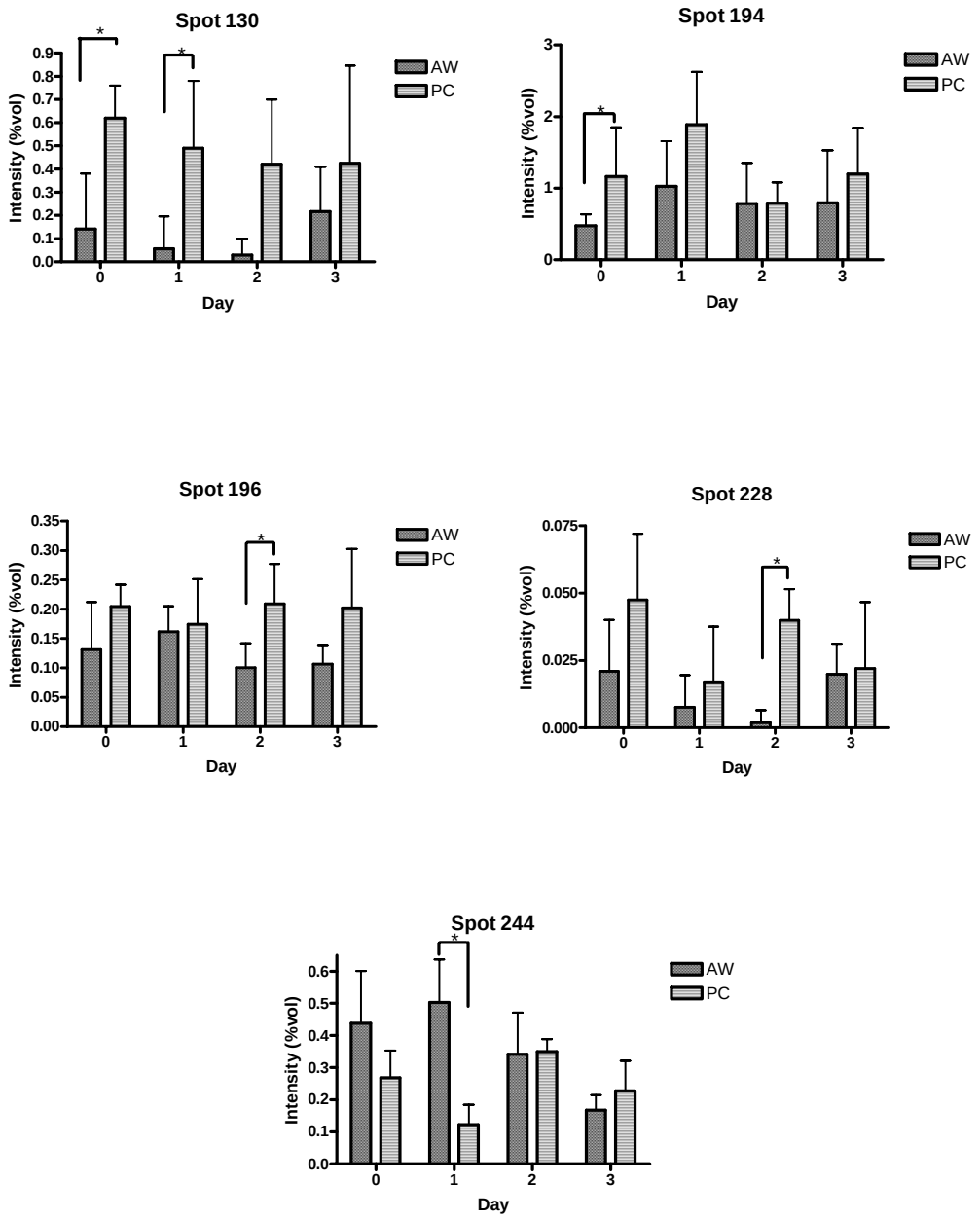


Figure 6.13: Protein spots which were significantly different when the AW and PC groups were compared. Each graph represents the intensity of one protein spot on days 0, 1, 2 and 3. Bars marked with * were significantly different (p-value < 0.05).

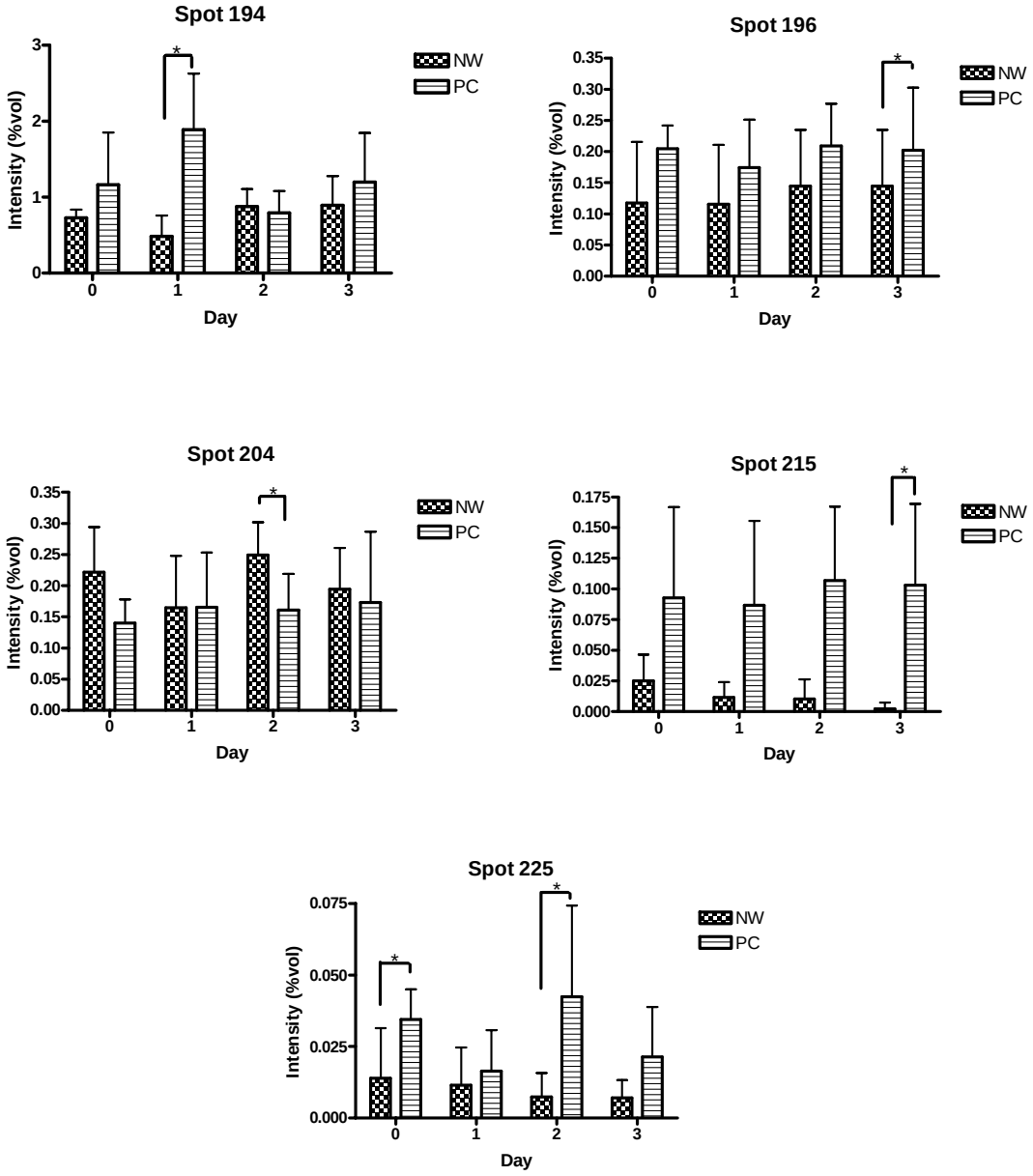


Figure 6.14: Protein spots which were significantly different when the PC and NW groups were compared. Each graph represents the intensity of one protein spot on days 0, 1, 2 and 3. Bars marked with * were significantly different (p-value < 0.05).

between day 1 and day 3. Spot 245 differed between day 2 and day 3 (Figure 6.15). Generally, the expectation of a control group is that it does not change over time. However, with regards to the serum proteome, proteins may have certain level changes due to natural rhythms or other bodily functions which affect these proteins. Therefore, to detect changes in protein levels in the NW group does not necessarily denote technical error or false statistical significance. It may, however, suggest changes unrelated to stress may occur. The reason for this control group was to determine the natural pattern of the serum proteome and, with the AW and PC groups, to look for a perturbation in this control pattern.

Five spots changed significantly during the trial within the PC group. Figure 6.16 presents these five spots and where the statistical difference lies. Spot 177 changed significantly between day 0 and day 3 and the intensity of spot 185 decreased significantly after day 0. Spots 216 and 234 were significantly different between day 0 and day 3 and day 0 and day 2 respectively. For spot 244, day 1 was significantly lower in intensity than days 0 and 2. There were more changes over the time course in the PC group than the NW group. This was somewhat expected since the PC group was weaned two weeks prior to day 0 and physiological changes associated with weaning might still be occurring.

The AW group displayed the most changes in serum proteins throughout the trial. Figure 6.17 reports all ten spots. Many of the changes in protein intensities within this group were very transient and could be due to random differences in protein levels. Such was the case for spots 130, 168, 170, 177, 196 and 197. For spot 137, there was a significant decrease in intensity after day 0 and this change remained constant for days

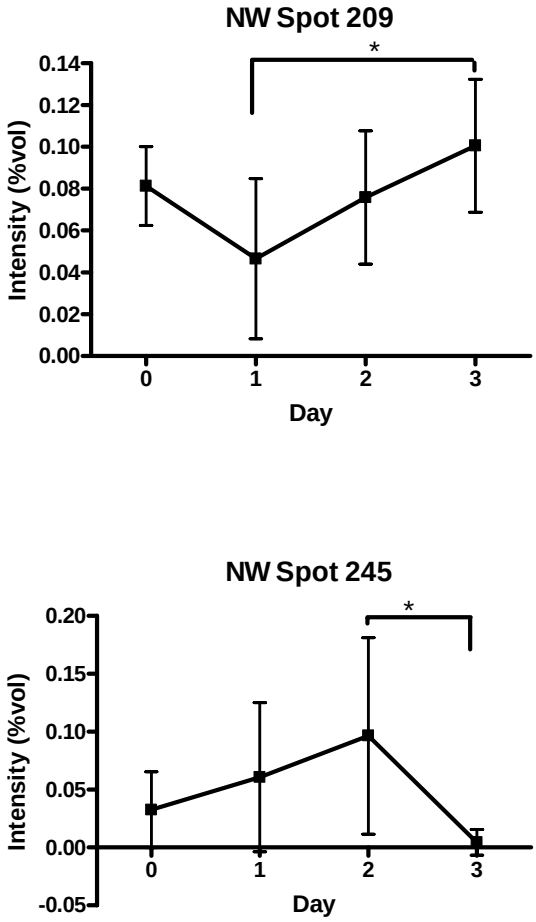


Figure 6.15: Spots which changed in intensity over the course of the trial in the NW group. Significant differences are marked with an *. The mean value plus/minus one standard deviation is shown.

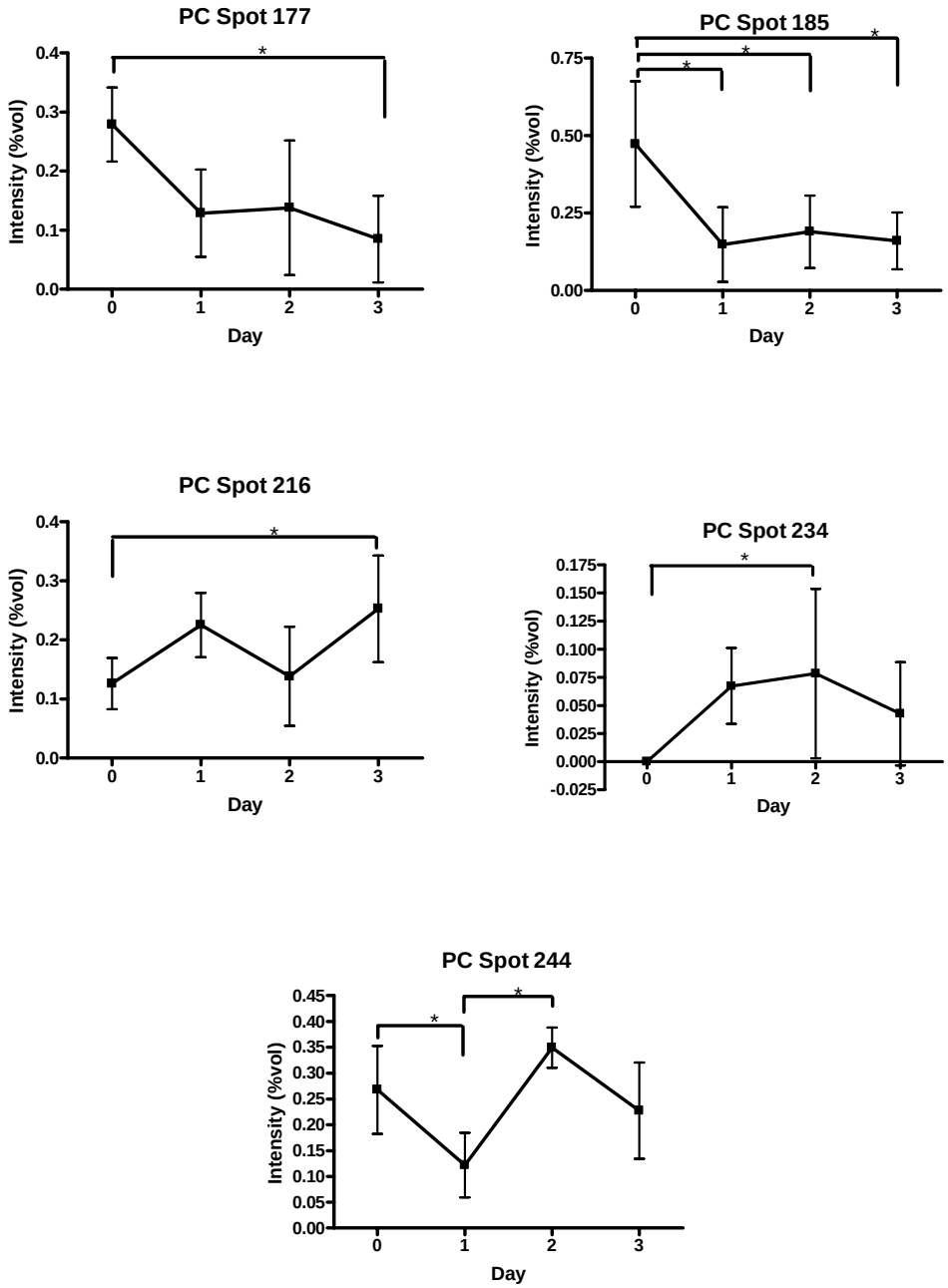
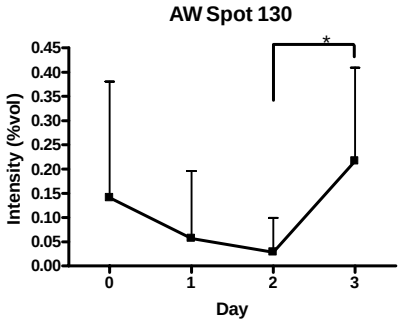
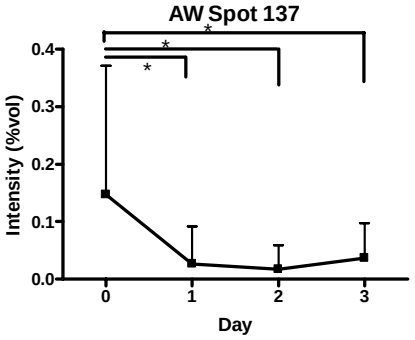
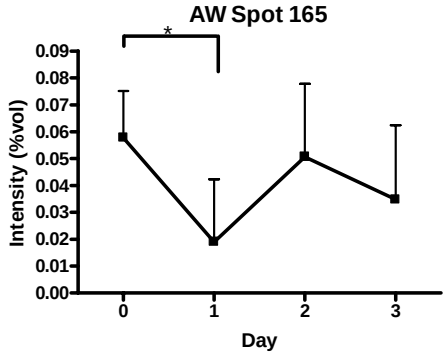


Figure 6.16: Changes in spot intensity over the course of the trial in the PC group. Significant differences are marked with an *. The mean value plus/minus one standard deviation is shown.

Figure 6.17: Changes in spot intensity over the course of the trial in the AW group. Significant differences are marked with an *. The mean value plus/minus one standard deviation is shown.


AW Spot 130
Intensity (%vol)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
1
2
3
Day
*


AW Spot 137
Intensity (%vol)
0.4
0.3
0.2
0.1
0.0
0
1
2
3
Day
*
*
*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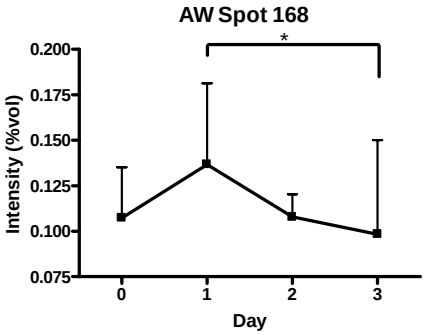
Intensity (%vol)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
1
2
3
Day
*


AW Spot 168
Intensity (%vol)
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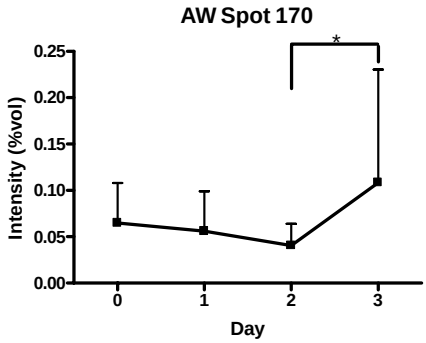
0.175
0.150
0.125
0.100
0.075
0
1
2
3
Day
*


AW Spot 170
Intensity (%vol)
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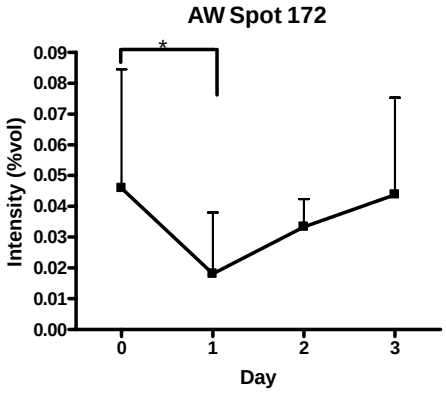
0.20
0.15
0.10
0.05
0.00
0
1
2
3
Day
*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Intensity (%vol)
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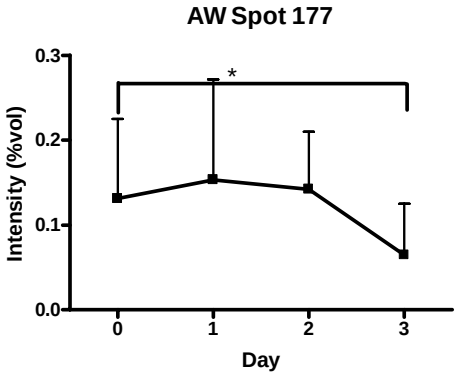
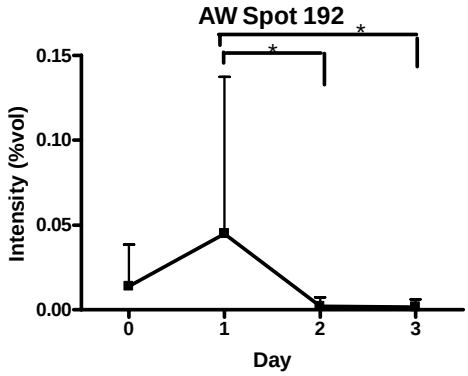
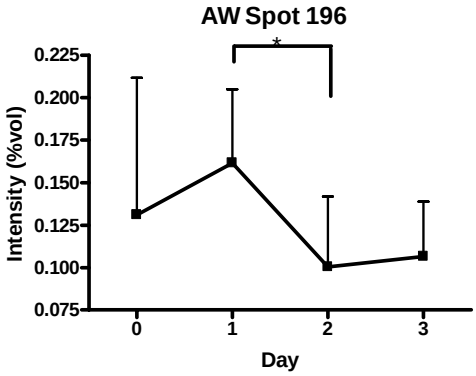
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
1
2
3
Day
*


AW Spot 177
Intensity (%vol)
0.3
0.2
0.1
0.0
*
0
1
2
3
Day


AW Spot 192
Intensity (%vol)
0.15
0.10
0.05
0.00
*
*
0
1
2
3
Day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Intensity (%vol)
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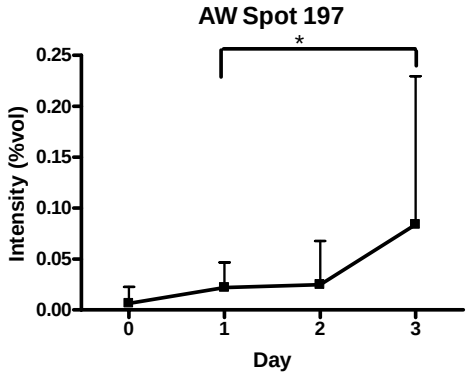
0.200
0.175
0.150
0.125
0.100
0.075
*
0
1
2
3
Day


AW Spot 197
Intensity (%vol)
0.25
0.20
0.15
0.10
0.05
0.00
*
0
1
2
3
Day

1, 2 and 3. Spot 192 also had a change in protein intensity which persisted for more than one day. Spots 165 and 172 both had a change in intensity between day 0 and day 1 which was consistent with the comparison between the groups in that the AW effects were seen mostly on day 1.

6.2.6 Identification of Proteins

Mass spectrometric analysis was performed on protein spots which were significantly different across the treatment groups. The search engine mascot was used to search the swiss prot database for matches to the mass spectrometry results. Table 6.1 shows the results from this mass spectrometric analysis. Table 6.2 shows the estimated molecular weights and isoelectric points of each spot mentioned in the results section.

Table 6.1 Identification of significantly different protein spots

Spot IDs	Predicted Protein Name
130	Alpha-1 acid Glycoprotein
187	Unknown
194	Unknown
196	Unknown
204	Unknown
215	Collagen alpha-1 precursor
225	Unknown
228	Unknown
244	Unknown

Table 6.2 Estimated molecular weights and isoelectric points of significant spots

Spot ID	Estimated Molecular Weight	Estimated Isoelectric Point
130	37	3.3
137	35	3.6
165	27	3.9
168	26	4.5
170	27	6.5
172	27	4.1
177	26	3.2
185	25	3.0
187	25	7.5
192	24	4.0
194	25	9.0
196	23	5.0
197	23	5.2
204	19	5.4
209	18	5.6
215	16	6.1
216	15	9.0
225	14	6.6
228	11	5.1
234	9	7.1
244	5	4.3
245	5	5.1

7.0 DISCUSSION AND CONCLUDING REMARKS

7.1 Trial 1: Method Validation

In order to address the hypothesis that weaning will cause sufficient stress in cattle to alter protein profiles in serum, traditional 2D-GE based proteomics techniques were chosen. The 2D-GE technique required optimization. A small trial was planned in order to determine whether changes due to DEX treatment were detectable and to provide samples to use when optimizing the methodology. When comparing a sample from a DEX treated animal with a control sample taken before DEX injection, we found that with the 2D-GE technique we could detect many proteins with differing abundances as well as changes in protein levels across samples. It was at this stage that a decision was made not to remove albumin from the serum samples. When albumin removal was performed following the manufacturers instructions of the SwellGel Blue albumin removal kit (Pierce Biotechnology; Rockford, IL), there was also removal of other proteins (data not shown). BSA functions as a transport protein, so the depletion of BSA may introduce artefacts through the removal of other proteins (Kragh-Hansen 1981). A kit for the specific removal of BSA has yet to be developed and optimized. In order to detect the most proteins and changes due to treatments, albumin was not removed from any serum samples analyzed. On the gel, BSA being larger in size remains at the top and does not interfere with the detection of lower molecular weight proteins. However, the presence of BSA does reduce the sensitivity for lower

abundance proteins. BSA being of high abundance reduces the amount of low abundance proteins being loaded into the gel since a fixed amount of total protein is used. With trial one, experience with the 2D-GE technique was gained and optimization of reagents took place. Changes in protein levels between a control sample and a treated sample were detected as well as changes in protein levels over the time course of the experiment were observed. The purpose of this trial was to determine whether changes in serum proteins due to a specific treatment could be detected and quantified.

7.2 Trial 2: Weaning stress

The objectives of this project were to determine:

a) The effects of abrupt weaning on the serum proteome by comparing AW calves to a control group which had never been weaned (NW).

b) The effects of abrupt weaning on the serum proteome by comparing AW calves to a control group which were previously weaned and allowed to recover (PC).

c) The effects of preconditioning calves on the serum proteome by comparing PC calves to a control group which were never weaned (NW).

d) The effects of abrupt weaning over the course of the trial.

The weaning stress trial was designed to test the hypothesis that weaning will cause sufficient stress in cattle to alter protein profiles in serum, which can then be used to identify this type of stress and to address the objectives. The trial consisted of three groups: an AW group, a PC group and a NW group. Body weight and temperature of each calf were monitored over the course of the trial. Body weight was measured to

determine if there was a significant difference in body weight gain among the three treatment groups. The AW group was expected to gain less weight than the PC group or the NW group, since these calves were experiencing weaning stress. Although the PC group had undergone weaning stress, this group represented either the possible long term effects of weaning or animals which had recovered from weaning stress. There was however no difference in weight gain when comparing among treatment groups. It has been reported that calves which are prevented from nursing and allowed to graze gain less body weight than calves who are nursing (Haley *et al.* 2005). Our results may have been due to measuring weights over a relatively short time interval during which the NW group did not gain a significant amount of weight. Perhaps monitoring body weight over a longer interval may have revealed significant differences in weight gain. A larger group size may be required to detect significant differences in weight gain due to weaning stress. Body temperature was also measured to determine if there were differences among the treatment groups and more importantly to monitor for possible illness. There were no significant differences among the groups and none of the calves developed a fever (body temperature > 40.2˚C for two consecutive days) during the trial.

Behavioral observations were also recorded for four animals from each treatment group. A wide range of behavioral responses were recorded but no statistical differences were observed among the three treatment groups. It is noteworthy; however, that the AW group was the only group to vocalize during the observation period. Monitoring more animals in each group might be required to detect statistical differences. This does not mean that there was no stress in the AW group but rather animal numbers were insufficient to detect the behavioral changes associated with

weaning stress. It is well established that weaning causes stress and changes in behavior in calves (Kelley 1980; Veissier *et al.* 1989; Watts *et al.* 2000; Haley *et al.* 2005).

Serum cortisol levels have frequently been used to monitor stress responses (Beerda *et al.* 1996; Grandin 1997; Queyras *et al.* 2004). It is thought that increased serum cortisol is an indicator of stress responses but some stressors do not induce a cortisolemia (Hickey *et al.* 2003). Serum cortisol levels also change very rapidly and it is sometimes difficult and impractical to sample sufficiently often to detect this spike in cortisol concentration (Queyras *et al.* 2004). For these reasons, cortisol may not be a reliable marker for stress. Cortisol was measured daily from serum sampled in the morning around the same time for all animals in each treatment group and the results (Figure 6.7) supported the conclusion that cortisol cannot be used to identify weaning stress. There were no significant differences in serum cortisol either within or among treatment groups. The AW group could not be distinguished from the two control treatment groups (NW and PC). This observation supports the conclusion that other markers for weaning stress are required. To increase reliability of stress detection it may also be better to use a pattern of several markers. In the present study, we were looking for a group of serum proteins which could be used to identify a weaning stress response.

The first objective of this project was to determine the effects of weaning on the serum protein profile of calves. To do this, a comparison was first made between an AW group and a control group which has never been weaned (NW). Through this comparison, there was a possibility of identifying altered protein levels which could be used to identify and possibly quantify a weaning stress response. The animal to animal

variation was high but, two proteins were found to have significantly different levels when comparing the AW group and the NW group. Both of these proteins may be good candidates as markers for the response to abrupt weaning. These proteins were spot ID 187 and 215. Both had differences detected on day 1 however spot ID 215 also was different on day 3. The concentration changes in these serum proteins on day 1 were unique to the AW group. The difference of 215 on day 3 was also apparent in the comparison of the PC group with the NW group. This may be an example of a long term effect of weaning since the PC group was weaned more than two weeks prior to day 3. These specific changes were apparent in Figure 6.12 and those on day 1 did not appear in samples taken from either the PC or the NW groups. Both possible marker proteins showed a change on day 1 post-weaning. This observation suggests that the effects of weaning on the serum proteins are most profound the first day after removal of the dams however these changes are very transient. Cortisol as a marker of stress also has a similar disadvantage.

To confirm the specificity of these stress biomarkers, the weaning trial should be repeated to determine if these two proteins consistently change following weaning stress. If these changes are confirmed then it may be possible to use these proteins to develop a diagnostic test to identify animals that are undergoing stress. Further work is required to determine the specificity of these markers to weaning stress as opposed to other types of stressors or physiological responses. To increase the effectiveness of identifying weaning stress, both markers would be required to make an accurate identification. This provides an advantage over cortisol in that there are two requirements for identification however both changes are still transient in nature. These

biomarkers might be useful in future weaning studies to determine if a link exists between stress and disease susceptibility. Once a stress response can be accurately identified in animals then it may be possible to study the connection between stress and disease.

A second objective for this weaning trial was to study the effects of PC animals in relationship to animals undergoing acute weaning stress. A comparison was made between the AW group and the PC group. The type of control group used in a study might influence which markers are identified to differentiate between treatments. This in fact was found to be the case in this study. All the protein markers for weaning stress when compared to the NW group were distinct from those when compared to the PC group. The markers were dependent on which type of control group was used. In terms of serum proteins, the PC group was also different from the NW group. Five proteins were found to be significantly different between the AW group and the PC group. There were more differences when comparing the AW group with the PC group than the NW group. It may be that long term changes due to weaning are different than those after acute weaning.

The third and final objective was to determine whether the PC animals had a protein profile which was similar to the NW animals. In other words, are these two types of controls interchangeable or are there serum proteins which may affect the analysis of serum protein profiles. Five serum proteins were found to be significantly different in intensity. These differences are scattered across all three days of the trial. These data supports the conclusion that the PC group had a serum protein profile which was distinct from the NW group. This suggests that there may be some long term

effects of weaning. PC groups have been used as control groups in weaning studies (Hodgson *et al.* 2005). The choice of this control group may have implications for the analysis of the data.

Statistical analysis was also performed to analyze further the proteins which changed within groups during the course of the trial. Ten proteins from the AW group were significantly different between two or more days of the trial. The PC group had 5 proteins which changed intensities over the course of the trial and the NW group had only two proteins which were significantly different between days of the trial. More changes over time were expected in the AW group than the other groups because it was expected to be undergoing the greatest changes. The NW group was a control group and served as a representation of normal occurrences in the serum protein profile if animals are not acutely stressed. Changes over time in this group correspond to natural changes in protein levels. Certain proteins, such as cortisol, are associated with a rhythmic pattern of secretion (Queyras *et al.* 2004). Perhaps the proteins changing over time in the NW group also have a pattern of concentration over time. Therefore, the serum protein profile of the NW group can change over time and this may not represent error in the 2D-GE technique. Changes over time in the control group complicate the comparison with an acutely stressed group. However, the NW group represents a control pattern and in order to find markers of weaning stress, perturbations of this control pattern are used. Any unique changes when compared to the control pattern can be used as a marker. One point to note is the fact that out of all the proteins found to be statistically different among the three treatment groups, only three of those proteins also differed in intensity during the course of the trial. Spot IDs 130, 196 and 244 are

proteins which both differ among the treatment groups and between the days of the trial within the same group. It is for this reason that there is more confidence in these proteins being true differences and not just variation. All differences noted in the results section were statistically significant but independent repetition in another trial would further increase confidence in these results.

Identification of proteins which were significantly different among the treatment groups was carried out by mass spectrometry. Only two proteins were identified. These proteins were 130 and 215. The low identification rate was most likely due to the lack of information in the bovine protein database. Although the bovine genome is completely sequenced, there was not as much information for bovine proteins as for human or murine proteins (D'Ambrosio *et al.* 2005). This makes identification of bovine proteins much more difficult. Protein ID 130 was identified as alpha-1-acid glycoprotein (AGP). AGP is an acute-phase protein (Fournier *et al.* 2000) and acute phase proteins increase or decrease in serum in response to injury, inflammation or infection (Ceron *et al.* 2005). This protein was significantly increased in the PC group on day 0 and day 1 compared to both the AW and NW groups. This suggests that a long term effect of weaning stress is an increase in AGP. Protein ID 215 was identified as collagen alpha-1 precursor. Collagen's function is mainly structural (Gelse *et al.* 2003). This collagen precursor was increased in the AW group on day 1 compared to the NW group. In the future, we may be able to identify a greater number of the significant proteins when the bovine protein database is more complete. This would provide a better understanding of the biology surrounding the changes occurring in response to weaning stress.

In conclusion, our hypothesis was correct. Abrupt weaning stress altered the serum protein profile of calves although the variation among animals was high within all groups. Many differences were also found between the protein profiles of calves undergoing abrupt weaning versus calves which had either undergone weaning (PC) or calves which have never been weaned. These differences were statistically significant and the pattern of differences between a control may be used to identify calves undergoing abrupt weaning stress. It was also found that different changes in serum proteins were identified depending upon which type of control group was used. Thirdly, there were many significant differences between the PC group and the NW group. This suggests that weaning had long term effects. The differences observed between the AW and the PC groups demonstrate that the long term effects are different from those observed after acute weaning. These two types of controls were different and this must be considered when planning a weaning study. These differences may have significant effects on the outcome of a weaning trial when proteomics methodologies are being used as an analytical tool. If differences in the serum protein profile exist, there may be differences in other molecules as well including metabolites. The type of control group used is important and should be considered carefully.

In the future, this trial should be repeated to confirm the specificity of these protein markers. The results of this trial may help to study weaning stress in relation to disease susceptibility. It is easier to study stress if it can be reliably identified. Perhaps a simple diagnostic tool could be developed to specifically identify and possibly quantify weaning stress in calves in other studies and potentially in the field. Weaning stress can have detrimental effects on cattle. If animals undergoing weaning stress could

be easily identified this may be used to design strategies to prevent disease susceptibility or infection. In addition, other common stressors in cattle may also be studied in this manner to determine whether the protein markers identified are unique to weaning stress or perhaps are broadly applicable to a variety of stressors. Weaning stress is a combination of psychological stress and physical (nutritional) stress. Perhaps the next step in weaning research is to study these two components separately to determine their relative contributions. The results of this trial are very important to the study of weaning stress and perhaps other stressors as well. This study has made a contribution to the understanding of effects of weaning stress on the serum proteome as well as identifying novel biomarkers of weaning stress. It has made a great contribution to the knowledge in this field and will be very useful in future weaning stress studies.

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